The Role of *BCLAF1* in Cellular Transformation

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SHPHEN003

Thesis presented for the degree of

Master of Science in Medicine

Medical Biochemistry

Date of Submission: 19th February 2018

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DECLARATION

I, Hendrina Mwiiwete Nelao Shipanga, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following people for all their support throughout the duration of this dissertation:

My supervisor, **Prof M.I. Parker**, for his continual support, guidance, knowledge and supervision.
My co-supervisor **Dr L.M. Mwapagha** for his willingness to help with techniques in the laboratory, his guidance and encouragement.
Members of Prof Parkers lab, past and present, **Dr Kevin Dzobo, Dimakatso Senthebane, Vimbaishe Chibanga** and **Dr Vuyolwethu Siyo** thank you for all the support and advice. You contributed to a very pleasant working environment.
Arielle Rowe, **Justin Henry** and **Reagon Peterson** for the general laboratory assistance.
My family and my friends for their support and prayers.

ABBREVIATIONS

APS	Ammonium persulfate
ATCC	American type culture collection
BCA	Bicinchoninic acid assay
BSA	Bovine serum albumin
***BCLAF1* siRNA**	siRNA targeting *BCLAF1*
***BCLAF1* KO**	*BCLAF1* knockout
btfL	*BCLAF1* long isoform
btfS	*BCLAF1* short isoform
Control siRNA	control non-targeting siRNA
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats/CRISPR-associated-9
crRNA	CRISPR RNA
cDNA	Complementary deoxyribonucleic acid
DAPI	4',6-diamidino-2-phenylindole
DDR	DNA Damage Response
DEPC	Diethylpyrococarbonate
DISC	Death-inducing signaling complex
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxy-Nucleotide Tri-Phosphate
DSB	Double Strand Break
EDTA	Ethylenediaminetetraacetic
FACs	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
FITC-Annexin V	Fluorescein isothiocyanate-Annexin V
gRNA	Guide RNA
γ-H2AX	phosphorylated H2AX
HCMV	Human cytomegalovirus

HIV	Human Immunodeficiency virus
INDELS	Insertions and deletions
KCl	Potassium chloride
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NHEJ	Non-homologous end joining
mRNA	Messenger RNA
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PI	Propidium Iodide
PKCδ	Protein Kinase c delta
P/S	Penicillin /Streptomycin
PBS	Phosphate Buffered Saline
RIPA	Radioimmunoprecipitation assay
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RNA	Ribonucleic acid
RNAi	RNA interference
RS domain	Arginine–Serine long repeats
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis
siRNA	short interfering ribonucleic acid
tracrRNA	trans-activating crRNA
TNFR	Tumor necrosis factor receptor
TBST	Tris-buffered saline and tween 20
UV	Ultraviolet

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ABSTRACT

The malignant transformation of normal cells into cancer cells result in the loss of control of cellular regulatory mechanisms such as loss of function of tumour suppressors and gain of function of oncogenes. Genetic mutations may be inherited or acquired during the process of malignant transformation, such that the normal mechanisms responsible for control cellular proliferation become dysfunctional.

Aberrations of the *BCLAF1* gene located on chromosome 6q23 has previously been detected by whole genome sequence analysis of DNA from oesophageal cancer biopsies. Although the role of *BCLAF1* is not well defined, some studies have shown *BCLAF1* to have functional connections linked to some of the known hallmarks of cancer such as cell proliferation, apoptosis and genome stability, thus linking *BCLAF1* to cellular transformation.

The objective of the study was to examine the effects of *BCLAF1* knockdown/*BCLAF1* knockout studies in cellular gene expression and tumorigenesis. *BCLAF1* expression was significantly reduced in the immortalized keratinocyte cell line (HaCaT), a lung transformed fibroblasts cell line (CT1), cervical cancer cell line (HeLa), breast cancer cell line (MDA-231) and two oesophageal cancer cell lines (KYSE30 and WHCO1), with a highly significant reduction in the breast cancer cell line (MDA-231). siRNA mediated knockdown of *BCLAF1* resulted in altered expression of several downstream genes including downregulation the proapoptotic genes *Caspase-3* and *BAX* and the DNA damage repair genes *EXO1*, *ATRIP* and *BACH1*. *BCLAF1* deficiency also attenuated *P53* expression and slightly increased *P21* expression resulting in a *P21*-dependent G1 phase cell cycle arrest. To our knowledge, this is the first study to identify H2AX as a downstream gene of *BCLAF1* that is downregulated by *BCLAF1* knockdown. Abrogation of *BCLAF1* in CT1 cells resulted in *P53*-dependent uncontrolled growth of cells, survival and accumulation of apoptosis-resistant cells, and genomic instability; all events that are crucial in tumourigenesis.

CHAPTER 1

Literature review

1.1 Introduction

Cancer is a malignant transformation of normal cells to cancer cells, an abnormal growth of cells that if not controlled and stopped could metastasize (Hahn *et al.*, 1999). Such malignant transformation results in loss of control of cellular regulatory mechanisms due to loss of function of tumour suppressors and gain of function of oncogenes (Jurel *et al.*, 2014). During malignant transformation, cancer cells accumulate genetic mutations that overpower the normal mechanisms that control cellular proliferation (Griffiths *et al.*, 2000). All cancer cells share common hallmarks that make them differ from neighbouring normal cells, these are 1. Stimulation and sustaining proliferative signalling, 2. Evasion of growth suppressors, 3. Evasion of apoptosis, 4. Enable replicative immortality; 5. Induce angiogenesis, 6. Activate invasion and metastasis, 7. Reprogramming of energy metabolism and 8. Evasion of the immune system (Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011).

The concept of 'Hallmarks of cancer' particularly guides translational research aimed at the improvement and development of biomarkers screening tests for early and rapid detection, precision medicine and quality of life for cancer patients (Hainaut & Plymoth, 2013). Single gene or multi-gene signatures are used as predictive biomarkers to measure specific molecular pathway deregulations in cancer (Goossens *et al.*, 2015). The gain of function in oncogenes disrupt the normal regulation of the cell signalling leading to deregulation of many cell pathways including cell cycle, apoptosis and invasion which eventually lead to cancer development (Giancotti, 2014). Cancer is associated with mutations in oncogenes and tumour suppressor genes which cause cells to undergo transformation leading to genomic instability (Albertson *et al.*, 2003). The changes in DNA sequence can be INDELS (insertions and deletions), duplications, genomic rearrangements, loss of heterozygosity in somatic cells or inherited mutated genes (Popova *et al.*, 2009). Detection of structural variations and patterns in genes contribute to cancer genome analysis and identification and discovery of oncogenes and tumour suppressor genes (Chin *et al.*, 2006; Liu *et al.*, 2015).

Tumour suppressor genes act in normal cells to suppress proliferation, by negatively regulating cell proliferation, and act through inhibition of the expression of a proto-oncogene or the activity of its protein (Marshall, 1991). Loss of function in tumour suppressors or the lack of these tumour suppressor genes in cancer cells exhibit uncontrolled cell growth associated with malignancy (Heaney & Melmed, 2005). In addition, abnormalities in tumour suppressor genes are the most frequently found in human tumours (Vogelstein & Kinzler, 1992; Soussi *et al.*, 2000). The identification of these abnormalities in tumour suppressors DNA has greatly facilitated the studies and understanding of human tumours and identification of specific genetic markers that are repeatedly reduced to homozygosity in several tumours of a type (Hahn & Weinberg, 2002).

BCLAF1 aberrations were detected through whole genome sequencing of DNA from biopsies of oesophageal cancer patients (Mwapagha, 2014). The deletion of *BCLAF1* had previously been shown to be associated with various cancers such as Non-Hodgkin's lymphoma, Burkitt's lymphoma and colorectal cancer (Kasof *et al.*, 1999; Lawrence *et al.*, 2014). *BCLAF1* was originally identified in a yeast two-hybrid screen of proteins that interact with E1B 19K, and subsequently shown as a protein-protein interacting partner with BCL2 family members, moreover, its overexpression induced apoptosis in HeLa cells and suppresses transformation by adenovirus E1A and E1B-19K proteins or mutant *p53* (Kasof *et al.*, 1999). Subsequent studies have suggested roles for *BCLAF1* in RNA metabolism processing (Bracken *et al.*, 2008; Sarras *et al.*, 2010), lung development and immune system functioning (McPherson *et al.*, 2009). Recent studies have shown that, depending on the extent of DNA damage, *BCLAF1* is involved in the γH2AX-mediated regulation of apoptosis and DNA repair, and is a γH2AX-interacting tumour suppressor (Lee, Yu, *et al.*, 2012). Moreover, *BCLAF1* is a key component of DNA damage induce-BRCA1 protein complex involved in mRNA splicing, DNA repair, cell-cycle arrest, and transcriptional regulation (Savage *et al.*, 2014). Taken together, these studies indicate that *BCLAF1* has functional connections and mutation patterns consistent with the known hallmarks of cancer that suggested *BCLAF1* contribution to cellular transformation (McPherson, 2012).

1.2 Structure of *BCLAF1*

BCL2-associated transcription factor 1 (*BCLAF1*), also known as BTF, or bK211L9 is a Bcl-2 family gene. The *BCLAF1* gene encodes 13 exons and is located on chromosome 6q23 where it spans 33 782 bases (McPherson, 2012). This region is reported to have a high occurrence of

deletions in different tumours, such as Non-Hodgkin's lymphomas, leukaemias and Burkitt's lymphoma (Merup *et al.*, 1998). The nuclear protein encoded by *BCLAF1* gene has four isoforms generated by alternative splicing with two predominant *BCLAF1* forms reported to be of 918 amino acids and of about 797-846 amino acids, missing 49 amino acids in length. The longer isoform with a molecular mass of 106 kDa and a smaller isoform with a molecular mass of 101 kDa (McPherson, 2012). *BCLAF1* localizes throughout the nucleus and slightly in the cytoplasm in a punctate-like manner and redistributes to the nuclear envelope loci in cells undergoing apoptosis. Its mRNA levels is ubiquitously expressed in skeletal muscle, haematopoietic cells, and various other cell lineages (Kasof *et al.*, 1999; McPherson *et al.*, 2009). Steady-state levels of BCLAF1 protein fluctuate in a temporal and cell-lineage dependent on the cell model during development (McPherson *et al.*, 2009).

BCLAF1 has been reported to be involved in various biological processes. *BCLAF1* was initially identified as interacting with the adenoviral homolog E1B19K. Overexpression of *BCLAF1* results in increased apoptosis and its transcriptional repression and apoptotic role in cells is reversible in the presence of *BCL2* or *BCL-XL* (Kasof *et al.*, 1999). However, *BCLAF1* does not exhibit any structural similarities with the BCL2 family proteins. *BCLAF1* has an N-terminal tract of Arginine–Serine long repeats (RS domain) (**Figure 1.1**) that is typically involved in pre-mRNA processing and is present in interchromatin granule clusters, nuclear substructures that act as repositories for mRNA splicing and transcription factors (Valcárcel & Green, 1996; Saitoh *et al.*, 2004). Although *BCLAF1* and antiapoptotic BCL2 members have been shown to have no common structural similarities, BCL2 protein interacts with BCLAF1 and prevent its localization to the nuclear envelope (Kasof *et al.*, 1999). Several studies have elaborated more on the link between *BCLAF1*, apoptosis and transcription (Sarras *et al.*, 2010). Protein kinase C δ (PKCδ) upregulates *P53* gene transcription through the core promoter element (CPE-TP53) in the *P53* promoter, *BCLAF1* is a trigger for protein kinase C δ-dependent *P53* gene transcription by interacting with CPE-TP53. Moreover, disruption of *BCLAF1*-mediated *P53* gene transcription leads to suppression of *P53*-mediated apoptosis in response to DNA damage (Liu *et al.*, 2007). Indeed, several functional links have been described for *BCLAF1* by different research groups in different biological processes, such as mRNA splicing and DNA repair (Lee, Yu, *et al.*, 2012; Savage *et al.*, 2014), senescence (Shao *et al.*, 2016), production of Kaposi's sarcoma-associated herpesvirus

(Ziegelbauer *et al.*, 2009) and human cytomegalovirus infection (Lee, Kalejta, *et al.*, 2012), lung development and T-cell activation (McPherson *et al.*, 2009).

1.3 BCLAF1 gene expression patterns in cancer

BCLAF1 protein is punctate-concentrated throughout the nucleus and its mRNA is prevalently expressed in skeletal muscle, hematopoietic cells, and many other cell lineages (Kasof *et al.*, 1999; McPherson *et al.*, 2009). BCLAF1 steady-state protein levels fluctuate from cell to cell in a mode-dependent manner during development. The *BCLAF1* locus at 6q23 has a high occurrence of deletions in various tumours, such as lymphomas and leukaemias (McPherson *et al.*, 2009), as well as in Raji cells, derived from Burkitt's lymphoma, which has deletions in 6q. In addition, *BCLAF1* aberrations were detected in oesophageal cancer biopsies through whole genome sequencing (Mwapagha, 2014). Two transcripts corresponding to *BCLAF1*; the shorter form, btfS and btfL, the longer isoform, both appear to be widely expressed but were deleted in some tumours (Kasof *et al.*, 1999). Moreover, the full-length isoform, btfL isoform, is significantly over-expressed in colon cancer cell lines and colorectal cancer tissue and is associated with elevated colony-forming ability, suggesting pro-tumorigenic ability for the L isoform in colon cancer cells (Zhou *et al.*, 2014). Post neoadjuvant therapy, both nuclear and cytoplasmic overexpression of *BCLAF1* occurs in rectal cancer and negative and weak nuclear *BCLAF1* expressions are independently linked with a poor prognosis of rectal cancer (Brown *et al.*, 2016).

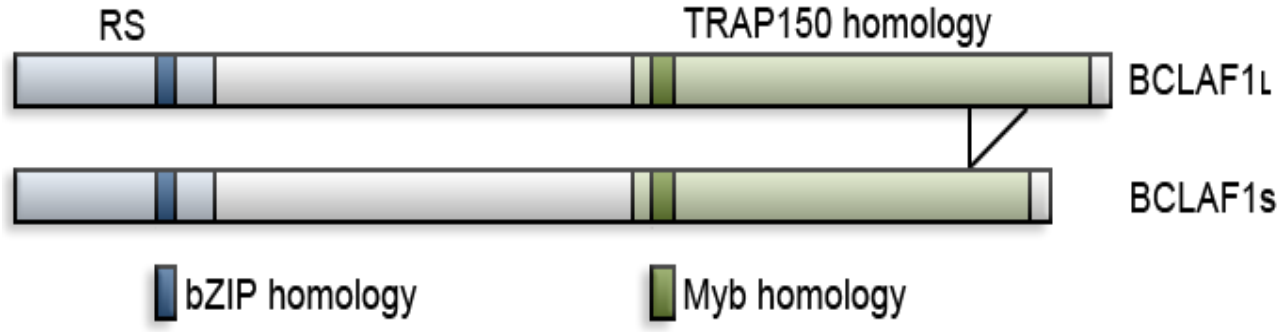

Figure 1.1. Protein domains in BCLAF1L and BCLAF1S isoforms. *BCLAF1* gene encodes for four splice variants, with two predominant BCLAF1 forms; BCLAF1L and BCLAF1S isoforms. The two predominant BCLAF1 forms, the longer isoform 920 amino acids in length with a predicted molecular mass of 106 kDa, and smaller isoform of 871 amino acids long with a predicted molecular mass of 101 kDa respectively. BCLAF1 has an N-terminal tract of Arginine–Serine long repeats (RS domain), a C-terminal region of thyroid hormone receptor associated protein 3 (THRAP3/TRAP150), a bZIP DNA binding domain and a Myb DNA binding domain. (McPherson, 2012).

1.4 Mechanisms of *BCLAF1* in Tumour Suppression

BCLAF1 operates via several mechanisms in its anticancer function involving apoptosis, cell cycle, DNA damage repair and genomic stability. In its anti-cancer role, *BCLAF1* is both directly and indirectly involved in the activation and stabilization of complexes involved in different signalling pathways. BCLAF1 co-localizes with γH2AX and stabilizes the Ku70/DNAPKcs complex which facilitates non-homologous end joining double strand breaks repair and induces the disassociation of anti-apoptotic Ku70/BAX complex in the cytoplasm, which liberates and activate proapoptotic BAX, promoting pro-apoptotic activity of Caspase-3 or Caspase-9 (Y. Y. Lee et al., 2012). *BCLAF1* is a key component of BRCA1-mRNA splicing complex involved in mRNA splicing and stabilization of genes such as *ATRIP*, *EXO1* and *BACH1* required for efficient DNA repair and maintenance of genomic stability (Savage *et al.*, 2014). *BCLAF1* initiates apoptosis in severely compromised cells through the induction of mitochondrial-mediated apoptotic pathway and by disturbing *P21*-mediated inhibition of Caspase/cyclin E-dependent apoptotic pathway (Kasof *et al.*, 1999; Lee, Yu, *et al.*, 2012). *BCLAF1* is essential for the DNA damage-induced senescence and it's an important NF-κB signal transducer (Shao *et al.*, 2016).

1.5 *BCLAF1* and the Hallmarks of cancer

1.5.1 *BCLAF1* in Apoptosis pathway

Apoptosis is a genetically controlled process of cell death which plays a key role in maintaining homeostasis and preventing the development of multiple diseases. Disruption and evasion of apoptosis leads to cancer (Kasof *et al.*, 1999; Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011). The BCL2 family consists of highly conserved proteins with different biological functions, many of the BCL2 members are the major regulators of apoptosis through multiple mechanisms (White, 1996). Overexpression of *BCLAF1* induced apoptosis in Hela cells (Kasof *et al.*, 1999). Recent studies indicate that *BCLAF1* induces apoptosis in various cell types (Shao *et al.*, 2016). Overexpression of *BCLAF1* induces a pro-apoptotic BAX activity. siRNA-mediated *BCLAF1* knockdown leads to reduced *BAX* expression, along with reduced activity of the *BAX*-downstream pro-apoptotic *Caspase-9* and *Caspase-3* (Sarras *et al.*, 2010). In addition, *BCLAF1* plays a role in disrupting the *P21*-dependent inhibition of activation of Caspase-dependent mitochondrial-mediated apoptotic pathways by negatively regulating *P21* expression in irradiated cells (Sarras *et al.*, 2010; Lee, Yu, *et al.*, 2012). In response to DNA damage-induced apoptosis, *BCLAF1* induces

the *P53*-mediated apoptosis by activating *P53* gene transcription through PKCδ-*BCLAF1* signalling (Liu *et al.*, 2007). Moreover, downregulation of *BCLAF1* is associated with Ku70-bound BAX complex formation in the cytoplasm, suggesting that *BCLAF1* is essential for the release of BAX from the anti-apoptotic Ku70–BAX complex (Lee, Yu, *et al.*, 2012) (**Figure 1.2A-B**).

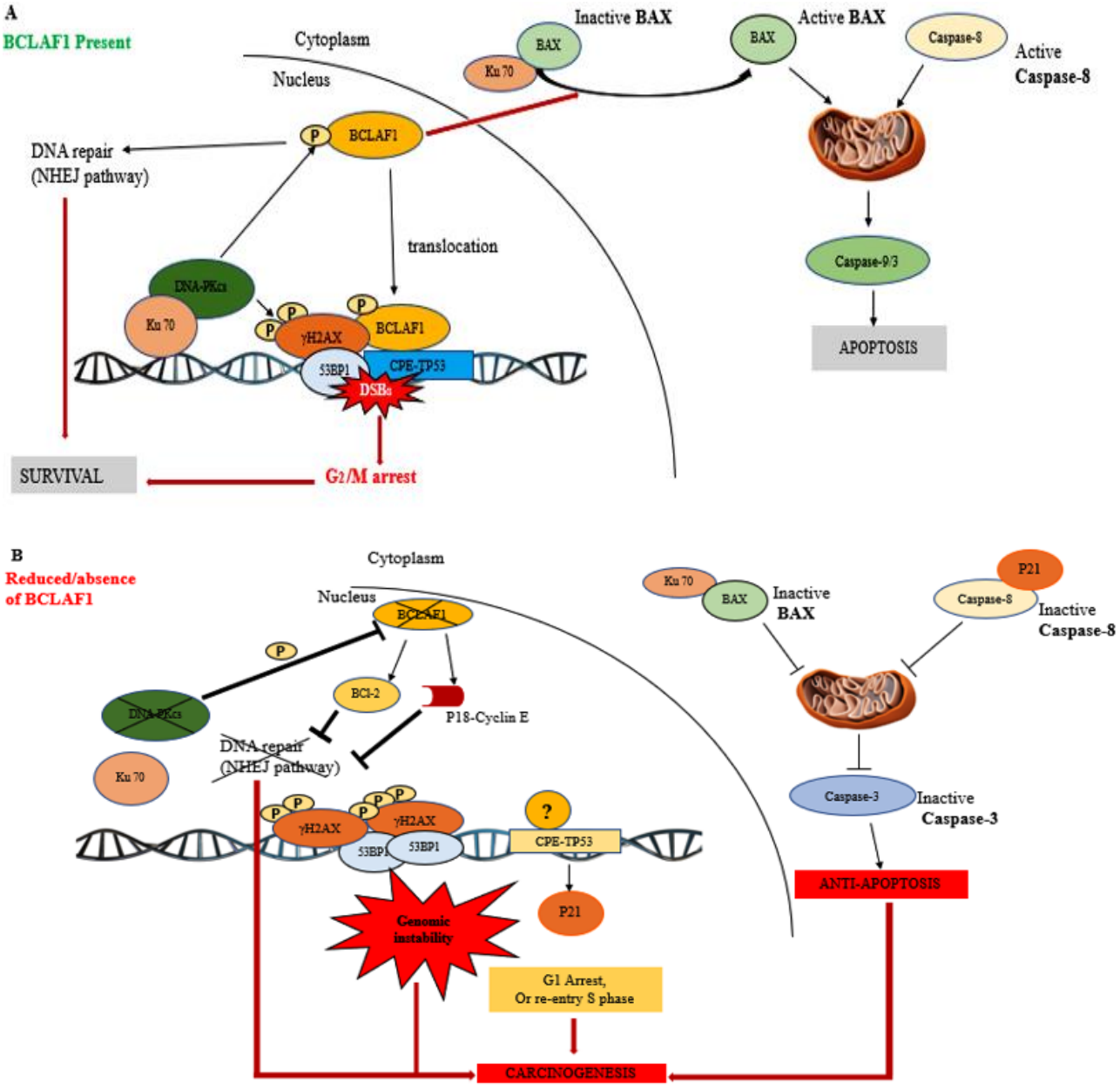

Figure 1.2A &1.2B. Mechanistic representation of various *BCLAF1* roles in various signalling pathways. In the presence of *BCLAF1*, following a site-specific phosphorylation of BCLAF1 by DNA-PKs induces the localization of BCLAF1 to the nuclear envelope, there it merges with γH2AX foci, which in turn stabilizes the Ku70/DNA-PKcs complex involved non-homologous end joining double strand break repair. Meanwhile in the cytoplasm, BCLAF1

induces the dis-association of Ku70/BAX complex, liberating active proapoptotic BAX. *BCLAF1* regulates both cyclin E and P53/P21dependent mitochondria-mediated pathways and inducing pro-apoptotic activity of Caspase-3. In contrast, in *BCLAF1* depleted cells, the Ku70/DNA-PKcs complex formation is weakened and depleted, subsequently reducing the activity of DNA repair contributing to cell survival with defective NHEJ DNA repair. Furthermore, there's inhibition of the phosphorylation of BCLAF1 by DNA-PKcs, hence, no translocation of BCLAF1 to the nucleus. In the cytoplasm, suppressed BCLAF1 results in more Ku70bound BAX, which inhibits the formation of pro-apoptotic BAX and pro-apoptotic Ku70-p18-Cyclin E complex. Modified from (Lee, Yu, *et al.*, 2012).

1.5.2 *BCLAF1* in DNA repair

Cells activate specific pathways for cell fate decisions depending on the severity of DNA damage. *BCLAF1* is involved in the γH2AX-mediated double strand DNA damage recognition and repair pathway (Lee, Yu, *et al.*, 2012). Through a site-specific phosphorylation of BCLAF1 by DNA-PKcs, BCLAF1 translocates to the nuclear envelope where it binds to γH2AX and regulates Ku70/DNA-PKcs complex stabilization which facilitate the non-homologous end joining (NHEJ) DNA double strand repair (**Figure 1.2**). Furthermore, *BCLAF1* positively regulate *P53* expression by interacting CPE-TP53 during DNA damage (Liu *et al.*, 2007). γH2AX accumulates DNA lesions in the nucleus, serving as the scaffold to temporarily assist *BCLAF1*, to access CPE-TP53, leading to upregulation of *P53* mRNA and protein levels in the irradiated cells (Lee, Yu, *et al.*, 2012). Subsequent studies indicated further roles for *BCLAF1* in DNA repair (Savage *et al.*, 2014; Orr & Savage, 2015). *BCLAF1* mediates the formation of a BRCA1-mRNA splicing-DNA damage-induced protein complex with other spliceosome proteins such as Prp8, U2AF35/65, SF3B1 through *BRCA1* mediated pathway which is involved in the regulation of mRNA splicing of several genes in response to DNA damage. BRCA1/BCLAF1-mediated mRNA splicing in response to DNA damage serves as a mechanism for the processing of key pre-mRNAs required for an effective DNA damage response and DNA repair. *BRCA1* and *BCLAF1* function in an epistatic manner within the same pathway. In addition, BRCA1/BCLAF1 mRNA splicing complex acts as a tumour suppressor complex, functioning to boost and promote the splicing and stability of genes required for DNA repair and maintenance of genomic stability. Depletion or loss of BRCA1/BCLAF1-mediated splicing of *ATRIP*, *BACH1*, and *EXO1* results in reduced but not abolished protein levels after DNA damage and in sensitivity to DNA damage and defective DNA repair (Savage *et al.*, 2014).

1.5.3 *BCLAF1* and the cell cycle

BCLAF1 is involved in *P21*-dependent cell cycle regulation. siRNA-mediated *BCLAF1* knockdown studies in human fibroblast cells attenuated *P53* expression but led to significant increases in *P21* expression, suggesting that *BCLAF1* is involved in *P21* regulation and induction *P21*-dependent G1 cell cycle arrest (Lee, Yu, *et al.*, 2012). Previous studies suggested that *BCLAF1* is a part of a complex that regulates *cyclin D1* mRNA stability (Bracken *et al.*, 2008). *Cyclin D1* and *cyclin E* are the essential components for regulating the cell cycle at either early G1 or late G1/S transition (Murray, 2004). *BCLAF1* knockdown suppressed the expression of *cyclin D1* (Lee, Yu, *et al.*, 2012), indicating that *BCLAF1* affects cell cycle progression by regulating the *P21*-dependent cyclins in early G1, which is often dysregulated in human cancers (Abbas & Dutta, 2009).

1.5.4 *BCLAF1* in mRNA splicing

BCLAF1 contains a Serine-Arginine rich region within its N terminus, present in many pre-mRNA processing factors and is involved in pre-mRNA processing and splicing (Ca´ ceres, 1997). *BCLAF1* forms part of a mRNA splicing complex together with SNIP1, SkIP, TAP150, and Pinin proteins which is required for the production of stable spliced *Cyclin D1* transcripts (Bracken *et al.*, 2008). *BCLAF1* is important in pre-mRNA splicing and interaction with various core splicing machinery proteins (Merz *et al.*, 2007). Savage and colleagues demonstrated that *BCLAF1* interacts with a number of other core mRNA splicing proteins such as Prp8, U2AF65, U2AF35, and SF3B1, within the BRCA1/BCLAF1 mRNA splicing complex. Independently of DNA damage, this interaction promotes efficient splicing and stability of several genes, such as; *ATRIP*, *BACH1*, and *EXO1* transcripts (Savage *et al.*, 2014; Orr & Savage, 2015).

1.5.5 *BCLAF1* role in T-cell activation and immune system functioning

Given the role of *BCLAF1* in apoptosis, McPherson and others demonstrated the critical functional role of *BCLAF1* in lymphocyte homeostasis, activation and the immune system. Although *BCLAF1* is non-essential for T cells development, it is required for maintenance of B- and T-cell homeostasis (McPherson *et al.*, 2009). In addition, depletion of *BCLAF1* results in the dysregulated activation of T cell proliferation but not B cells, and a significant decrease in T cells in the S-phase and an increase at the G1 and G2/M phases of the cell cycle as compared to the *BCLAF1* wild type.

Thus confirming that *BCLAF*1 is required for proper proliferation and activation of T lymphocytes (McPherson *et al.*, 2009; Kong *et al.*, 2012).

1.5.6 Other *BCLAF1* roles

Besides the functional roles of *BCLAF1* in cancer, various researchers investigated the roles of *BCLAF1* in other processes such as skeletal muscle differentiation, smooth muscle and lung development (McPherson *et al.*, 2009; Sarras *et al.*, 2010; Sarras, 2012). *In vivo* studies on the analysis of *BCLAF1* deficiency on muscle differentiation indicated that *BCLAF1* deficiency reduced the number of myoblastic cells in muscle compared to *BCLAF1* wild type controls (Sarras, 2012), although the whole concept is not well understood. Furthermore, *BCLAF1* expression is significantly upregulated during the saccular stage of mice embryonic lung development and surprisingly, *BCLAF1* deficient mice primarily die as neonates with defects in end-stage lung development that compromise survival (McPherson *et al.*, 2009).

1.6 *BCLAF1* deregulation is a hallmark of tumorigenesis

BCLAF1 is intrinsically suppressed in tumour cells with natural radio-resistance (Lee, Yu, *et al.*, 2012). Mutational inactivation of *BCLAF1* occurs in different tumours and various biological processes concomitant with tumorigenesis. Deregulation or inactivation of *BCLAF1*-proapoptotic and DNA repair factors contribute to apoptotic resistance and cell survival with defective DNA repair which is associated with tummourigenesis (Lee, Kalejta, *et al.*, 2012). Various *BCLAF1* knockdown mechanisms indicate that the downstream effects of *BCLAF1* deficiency is similar to mutant *BCLAF1* effects, including reduced apoptotic cells (Kasof *et al.*, 1999; Sarras *et al.*, 2010; Lee, Kalejta, *et al.*, 2012), reduced frequency of *BCLAF1* translocation to the nuclear envelope affecting the γH2AX-BCLAF1 complex activity (Lee, Kalejta, *et al.*, 2012), moreover, tumour suppressor BRCA1/BCLAF1 mRNA splicing complex activity is supressed (Savage *et al.*, 2014), *P21*-dependent cell arrest at G1 phase or re-entry to S phase with replicating the errors caused by DSBs (Lee, Kalejta, *et al.*, 2012) and all events that are crucial in tumourigenesis .

1.7 Clinical and therapeutic implications of *BCLAF1* dysfunction

BCLAF1 plays a critical role in apoptotic signalling (Kasof *et al.*, 1999). Apoptosis crucial in the efficient removal of damaged cells such as those occurring after DNA damage or during development and cell homeostasis (Fuchs & Steller, 2011). Apoptosis can be used as a vehicle for targeted treatment in cancer and deregulation of *BCLAF1* expression and cell death signalling is

associated with uncontrolled cell proliferation, development and progression of cancer (Pistritto *et al.*, 2016). Understanding the apoptotic mechanisms and therapeutic strategies co-targeting molecules involved in apoptotic resistance can be used to restore cancer cell sensitivity to apoptosis and overcome the ineffectiveness of certain drugs (Giménez-Bonafé *et al.*, 2009). The critical role of *BCLAF1* contributes to effective maintenance of DNA integrity and genomic stability (Lee, Kalejta, *et al.*, 2012; Savage *et al.*, 2014). The *BCLAF1* interacting partner THRAP3 functions together in the BRCA1/BCLAF1 complex to promote the splicing of precursor mRNA and export of target transcripts (Savage *et al.*, 2014). Mutant BCLAF1/THRAP3 in tumours could be targets for DNA damaging chemotherapy (Vohhodina *et al.*, 2017).

1.8 The use of RNAi technology and CRISPR/Cas9 system for gene function validation

RNA interference (RNAi) and clustered regularly-interspaced short palindromic repeats (CRISPR)-associated 9 (CRISPR/Cas9) are powerful genetic tools for validating gene function in biological science research and provides a promising therapeutic modality for cancer and other genetic diseases (Guo *et al.*, 2013; Haussecker, 2016). Both RNA interference and CRISPR/Cas9 technologies are RNA-guided mechanisms that cause the cleavage at targeted loci in the genomic DNA sequence complementary to their specific sequences and they can be applied in specific biological processes *in vivo* and *in vitro* (Guo *et al.*, 2013; Haussecker, 2016). With the advent of large-scale gene knockdown and knockin/knockout using siRNA libraries and CRISPR/Cas9 respectively, it has become possible to quickly identify novel drug targets as well as explore their roles in tumorigenesis (Morgan-Lappe *et al.*, 2006; Nickles *et al.*, 2012; Boyer *et al.*, 2013; Radia *et al.*, 2013; Sánchez-Rivera & Jacks, 2015; Khan *et al.*, 2016).

Even though functional connections and mutation patterns of *BCLAF1* consistent with the known hallmarks of cancer are known, the molecular role and contribution of *BCLAF1* in tumourigenesis remain unclear. siRNA-mediated *BCLAF1* knockdown and CRISPR/Cas9-mediated *BCLAF1* knockout studies were done in transformed human lung fibroblast CT1 cell line to examine the role of *BCLAF1* knockdown and deletion in tumourigenesis.

1.9 Objective of the study

Whole genome sequencing studies of oesophageal cancer biopsies have shown *BCLAF1* aberrations in oesophageal cancer (Mwapagha, 2014). Furthermore, *BCLAF1* deletions have been observed in a number tumour types, and very little is known about *BCLAF1* expression patterns in

human cancers and its deficiency/presence effects on gene expression, and tumourigenisis (Kasof *et al.*, 1999; Zhou *et al.*, 2014). The main objective of the study was to look at the effects of *BCLAF1* knockdown/knockout studies in fibroblast cells on cellular gene expression and tumorigenesis.

1.10 Aims of the study

1. Expression patterns of *BCLAF1* in different cancer cell lines.

2. Effects of targeted siRNA-mediated *BCLAF1* knockdown and CRISPR/Cas9-mediated *BCLAF1* knockout studies on cellular gene expression.

3. Effects of targeted siRNA-mediated *BCLAF1* knockdown and CRISPR/Cas9-mediated *BCLAF1* knockout studies on apoptosis and DNA repair.

CHAPTER 2

Expression of *BCLAF1* in human tumour cell lines

2.1 Introduction

The insight into cancer molecular biology has been revolutionized by next generation sequencing technologies. Next generation sequencing provides cancer gene discovery and identification of specific mutations relevant to cancers and other genetic diseases reviewed in (Yadav *et al.*, 2015). *BCLAF1* is located on chromosome 6q23, a region reported to have a high occurrence of deletions in various cancer types including lymphomas and leukaemias (McPherson *et al.*, 2009). In addition, cancer associated aberrations have been found in several genes located in this region including *BCLAF1*, suggesting a role in carcinogenesis (Kasof *et al.*, 1999; Mwapagha, 2014). Two transcripts of *BCLAF1*, the shorter and the longer isoform, btfS and btfL respectively are widely expressed but deleted in several tumours (Kasof *et al.*, 1999), moreover, the full-length isoform (btfL), is significantly over-expressed in colon cancer cell lines and colorectal cancers and is associated with elevated colony-forming ability, suggesting pro-tumorigenic ability for the L isoform in colon cancer (Zhou *et al.*, 2014).

Several studies suggested a role for *BCLAF1* in different signalling pathways, and a number of *BCLAF1*-associated genes have been identified to date. Over expression of *BCLAF1* in HeLa cells induces apoptosis (Kasof *et al.*, 1999). Subsequent studies investigated the role of *BCLAF1* role in DNA repair (Lee, Yu, *et al.*, 2012; Savage *et al.*, 2014; Shao *et al.*, 2016; Vohhodina *et al.*, 2017). Lee and colleagues identified BCLAF1 as a H2AX associated protein involved in the regulation of γH2AX-mediated regulation of DNA repair Ku70/DNA-PKcs complex stabilization and efficient DNA repair (Lee, Yu, *et al.*, 2012). Furthermore, *BCLAF1* is a crucial component of the BRCA1/BCLAF1 mRNA splicing complex that is implicated in the stabilization and efficient production of transcripts of various genes such as *EXO1*, *ATRIP*, and *BACH1* that are involved in DNA repair independently of DNA damage (Savage *et al.*, 2014). *BCLAF1* is involved in the regulation of the cell cycle by upregulating *P21* expression in *BCLAF1*-deficient cells, resulting in a G1 phase cell cycle arrest (Lee, Yu, *et al.*, 2012).

This chapter examined *BCLAF1* expression patterns in a variety of human cancer cell lines and the expression of genes linked to *BCLAF1* in different cancer signalling pathways such as apoptosis, DNA damage response (DDR) and cell cycle.

2.2 Results

2.2.1 *BCLAF1* mRNA and protein expression in cultured transformed and cancer cell lines

BCLAF1 expression was examined in gamma radiation transformed human lung fibroblasts (CT-1), SV40 immortalized keratinocytes (HaCaT); a cervical cancer cell line (HeLa), a breast cancer cell line, (MDA-231) and two oesophageal cancer cell lines (KYSE30 and WHCO1). *BCLAF1* expression in these cell lines was determined by measuring mRNA by real-time polymerase chain reaction (RT-PCR) and protein by western blotting.

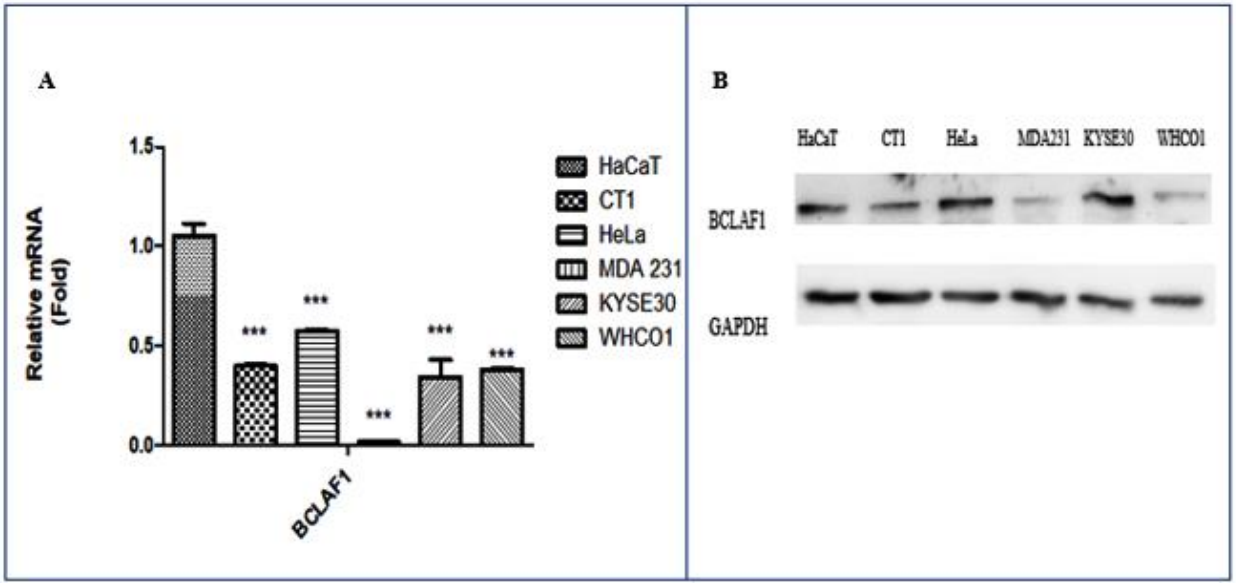

Figure 2.1. *BCLAF1* mRNA and protein expression in different human cell lines. A); RT-PCR analysis of *BCLAF1* gene expression using RNA extracts from the indicated cell lines and *BCLAF1* specific primers (Table 5.5) to detect *BCLAF1* mRNA expression. B); Western blot analysis of BCLAF1(106kDa) and GAPDH (37kDa) (used as a loading control) in protein extracts prepared from the indicated cell lines as described in Materials and Methods.

Analysis of *BCLAF1* mRNA by RT-PCR analysis showed *BCLAF1* mRNA expression to be significantly lower in all the cancer cell lines and an exceptionally low level of expression was observed in MDA-231 cell line (**Figure 2.1A**). The same low levels of BCLAF1 protein were observed in the MDA-231 and WHCO1 cell lines (**Figure 2.1B**). BCLAF1 protein's half-life is shorter in the WHCO1 cells than the KYSE30 cells, which could explain the unrelated results.

2.2.2 Genes associated with *BCLAF1*

We further investigated the mRNA profiles of genes linked to *BCLAF1* in different signalling pathways such as apoptosis, DNA repair and cell cycle regulation in the same cell lines. Firstly, genes involved in the apoptosis signalling pathway, the proapoptotic genes such as *BAX*, *Caspase-3*, and *Caspase-9* as well as anti-apoptotic BCL2 family members; *BCL2*, and *BCL-XL* mRNA expressions were examined, following DNA damage repair signalling pathway genes; *EXO1*, *BACH1*, *ATRIP*, *Ku70*, *H2AX* and *BRCA1*. We assessed mRNA profiles of cell cycle regulators; *P53* and *P21* in the mentioned cell lines.

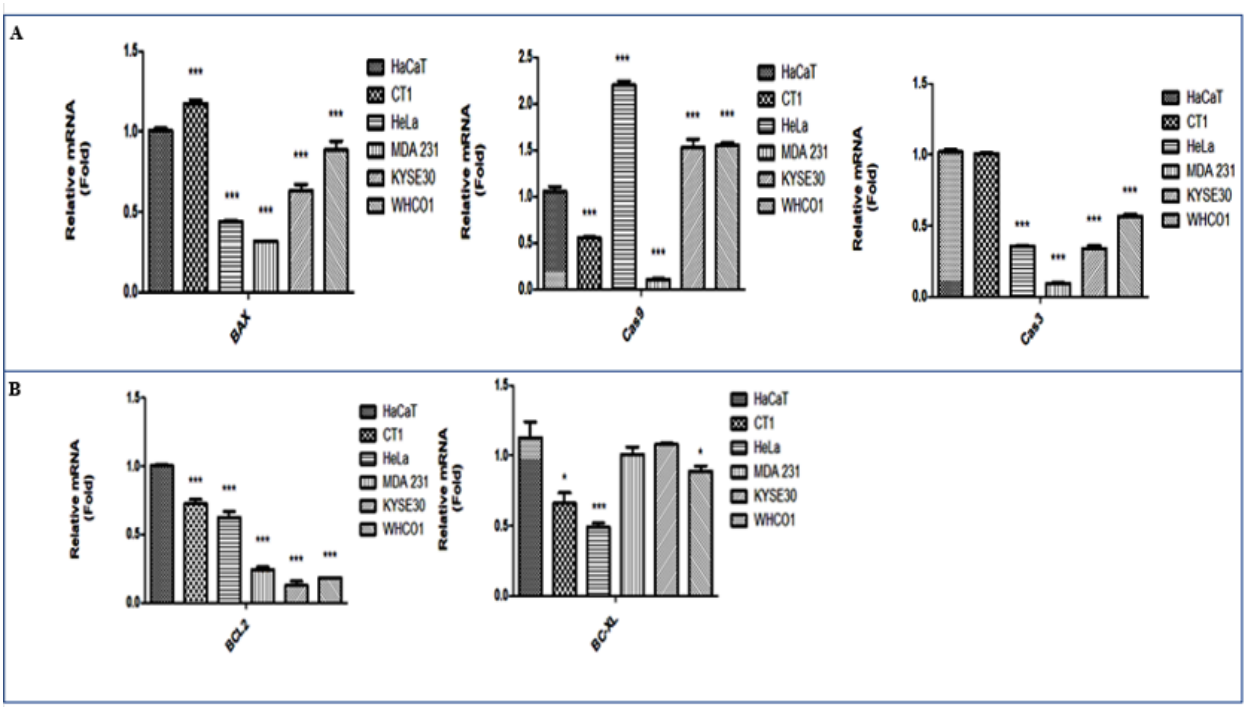

Figure 2.2. Proapoptotic and anti-apoptotic gene expression in cultured cell lines. RNA was extracted from the transformed cell lines; HaCaT and CT1, cancer cells lines; HeLa, MDA231, KYSE30 and WHCO1 as described in Material and Methods and used in RT-PCR analysis for proapoptotic and anti-apoptotic mRNA levels in the indicated cell lines. The specific primer pairs used for each of the selected genes are indicated in Table 5.5. A); Analysis of the proapoptotic *BAX*, *Caspase-3* and *Caspase-9* genes. B); Analysis of anti-apoptotic *BCL2* and *BCL-XL* genes.

Analysis of proapoptotic (**Figure 2.2A**) and antiapoptotic (**Figure 2.2B**) mRNA expression by RT-PCR analysis showed proapoptotic *BAX* and *Caspase-3* genes to be reduced in all the cancer cell lines, but not the transformed cell lines, while extremely low levels of all three proapoptotic gene transcripts were observed in the MDA-231 cell line. Anti-apoptotic *BCL2* mRNA levels is

low in all cancer and transformed cell lines, in contrast, not much difference was observed in *BCL-XL* mRNA expression in MDA-231 and KYSE30 cell lines.

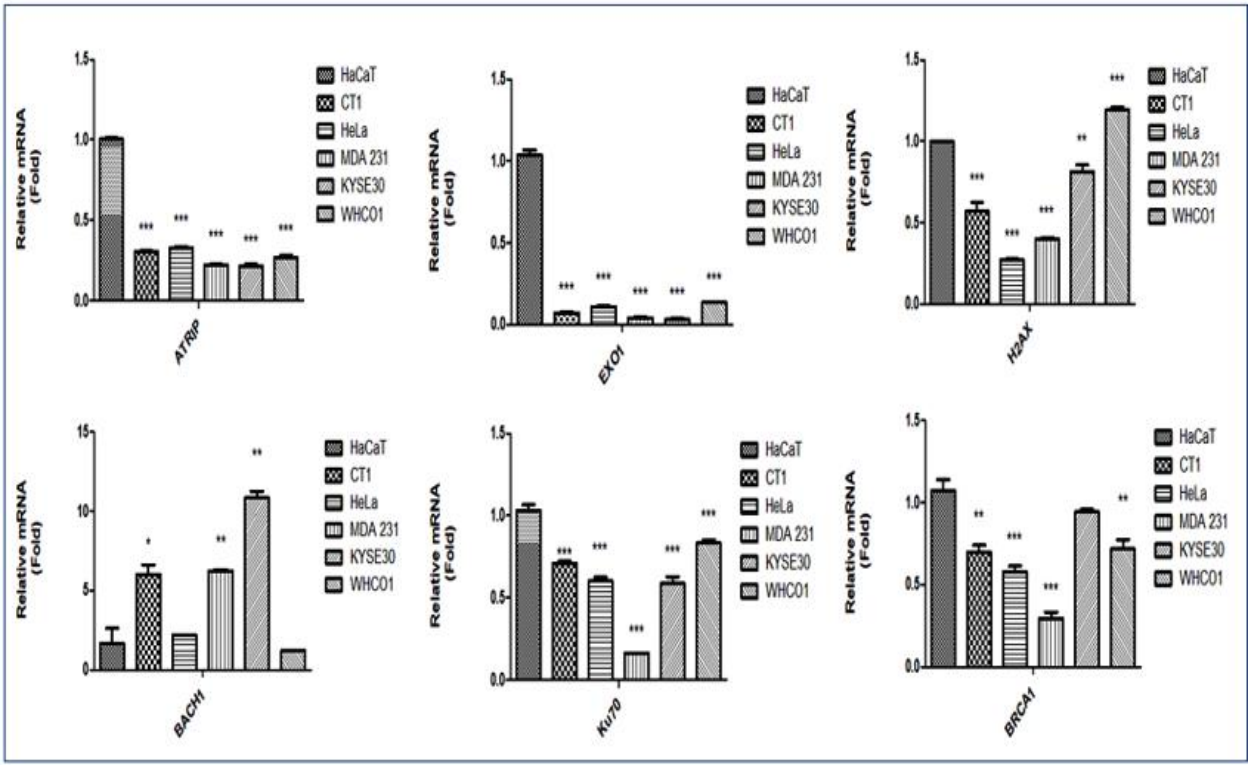

Figure 2.3. DDR genes mRNA expression in cultured cell lines. RNA was extracted from the cells lines as described in Material and Methods and used in RT-PCR analysis for genes involved in DDR mRNA levels in the indicated cell lines. The specific primer pairs used for each of the selected genes are shown in Table 5.5.

Mismatch repair and recombination gene *EXO1*, DNA double-strand break repair genes i.e. *ATRIP*, *Ku70* and *BRCA1* showed significantly low mRNA expression in all cancer cell lines, furthermore, extremely low levels of *Ku70, BRCA1* gene transcripts were observed in the MDA-231 cells, in contrast *BACH1* was differentially expressed across the cell lines (**Figure 2.3**).

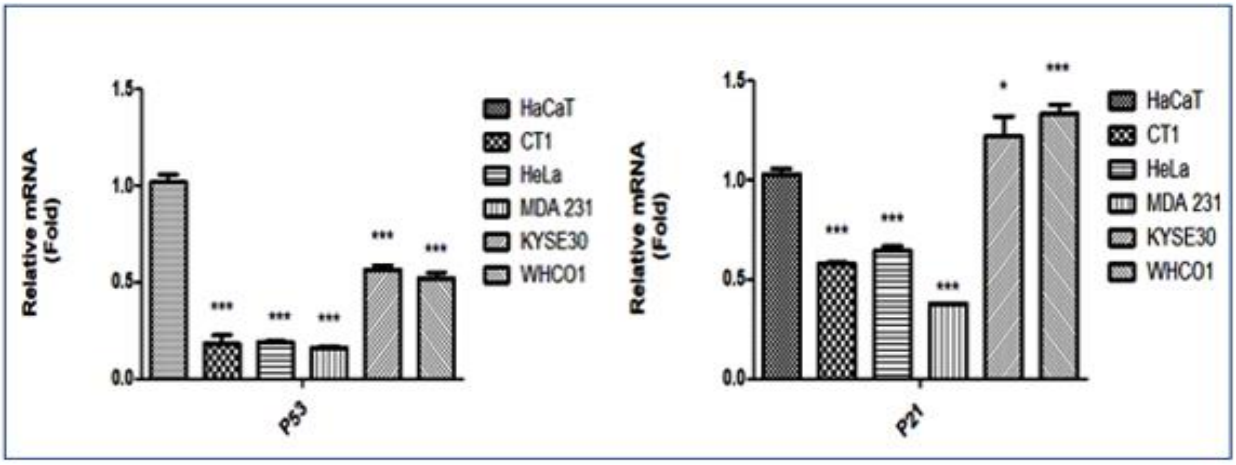

Figure 2.4. Cell cycle regulators mRNA expression in cultured cell lines. RNA was extracted from the cells lines as described in Material and Methods and used in RT-PCR analysis for *P53* and *P21* mRNA levels in the indicated cell lines. *P53* and *P21* mRNA was quantitated using specific primers as indicated in Material and Methods was quantitated using primer pairs as indicated in Table 5.5.

Analysis of cell cycle regulators mRNA expression by RT-PCR analysis (**Figure 2.4**) showed reduced *P53* mRNA levels in all the cancer cell lines compared to immortalized keratinocytes HaCaT cells. *P21* gene transcript was significantly high expressed in all oesophageal cancer cell lines; KYSE30 and WHCO1 but reduced in CT1, Hela and MDA-231 cells. Due to the observations and conclusion on the effects of BCLAF1 deficiency on the expression of P53 and P21, i.e., knocking down BCLAF1 attenuated P53 expression both at RNA and protein levels, and slightly upregulated P21 mRNA and protein levels. Comparing the ratio of P53/P21 levels in the immortalized cells versus the cancer cell lines, one could conclude on the results in Figure 2.4, that the deficiency BCLAF1 levels in the cancer cell lines contributed to the attenuated P53 levels and upregulation of P21 expression.

2.2.3 Cell cycle analysis

Given the involvement of *BCLAF1* in *P53* transcription activation and in the γH2AX-mediated regulation of P53/P21 expression and cell cycle progression (Liu *et al.*, 2007; Lee, Yu, *et al.*, 2012). Flow cytometric analysis was conducted to assess the cell cycle flow of the cell lines. CCD1068SK is a normal breast cell line.

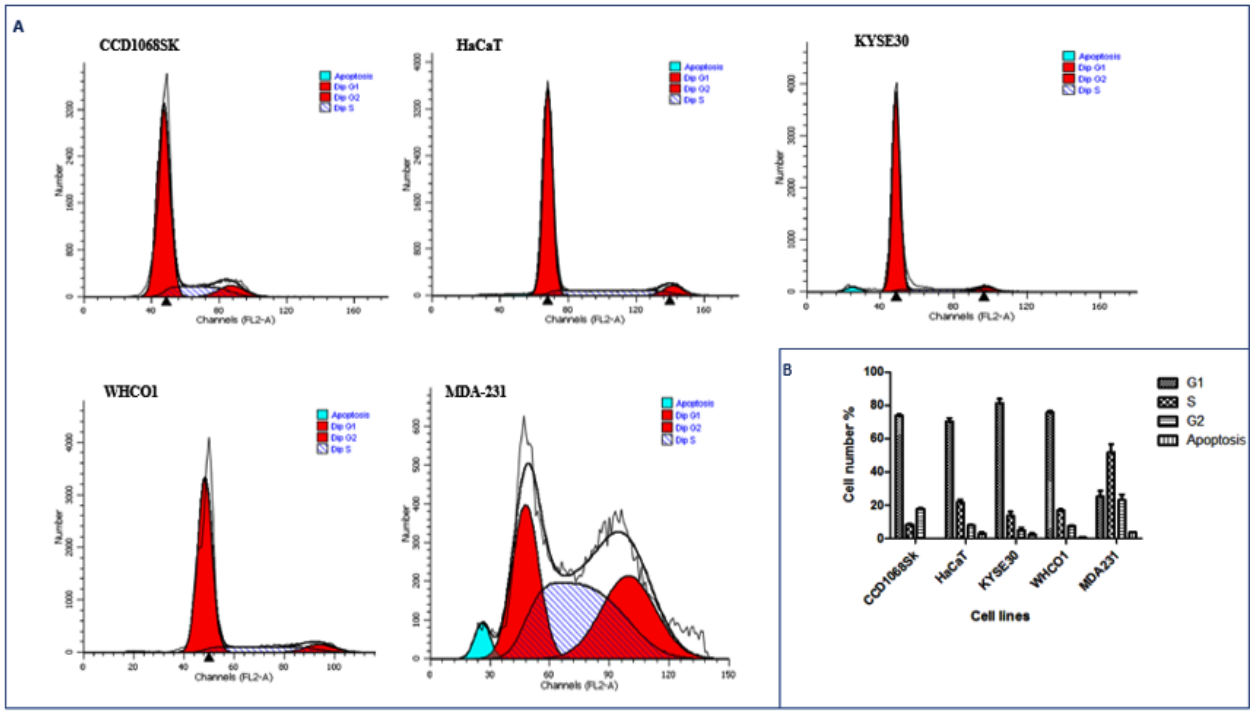

Figure 2.5. Flow cytometry analysis of the cell lines cell cycle progression. Flow cytometry analysis of CCD1068SK, HaCaT, MDA-231, KYSE30 and WHCO1 cells was carried out as described in Material and Methods. Cells were stained with propidium iodide (PI) and analysed using a FACScan cell sorter. **A**); Each cell line was conducted in triplicate and 20 000 events were captured. **B**); The bar graphs showed the relative quantity of G1, S, G2 and Apoptotic cells population in the five cell lines analysed.

Cell cycle progression of CCD1068SK, HaCaT, MDA-231, KYSE30 and WHCO1 cells was conducted by flow cytometry analysis (**Figure 2.5**). MDA-231 cells showed higher cell population of the S-phase, slightly reduced G1 cell population and more apoptotic cells compared to the other four cell lines, with more cell population in the G1 phase.

2.3 Discussion

Several genetic variations in the human genome are known to play a significant role in tumourigenesis. Chromosomal translocations, loss of heterozygosity, insertions and deletions are some of the large scale mutational events in the DNA sequence of a gene (Talseth-Palmer & Scott, 2011). Whole genome sequencing studies of oesophageal cancer biopsies have shown *BCLAF1* aberrations in oesophageal cancer (Mwapagha, 2014).

Although *BCLAF1* deletions have been observed in a number tumour types, very little is known about *BCLAF1* expression patterns in human cancers or in cancer cell lines. In addition, several BCLAF1 splice variants have been identified and have been observed to be differently expressed in different tumour types (Kasof *et al.*, 1999; Zhou *et al.*, 2014). This study examined at the expression patterns of *BCLAF1* in different human transformed and cancer cell lines as well as genes linked to *BCLAF1*.

BCLAF1 was found to be expressed in low levels in all cancer cell lines and at extremely low levels in MDA-231 and WHCO1 cells. Even though mutant *BCLAF1* is not associated with breast cancer, low *BCLAF1* mRNA levels observed in the triple-negative breast cancer MDA-231 cells might contribute to the tumour development or better yet contribute to the classification and treatment of the breast cancer tumours in triple negative breast cancer. *BCLAF1* is a restriction factor of oncogenic-human cytomegalovirus (HCMV). Elevated expression of *BCLAF1* inhibits HCMV replication, and, hence suppressing and preventing HCMV infection. Moreover, BCLAF1 steady-state protein levels are degraded by HCMV immediately following its infection through two temporally independent mechanisms identified by (Lee, Kalejta, *et al.*, 2012) work. HCMV targets a number of different cell types, including, epithelial cells, endothelial cells, fibroblasts, stromal cells, and hepatocytes (Herbein & Kumar, 2014). Although HCMV has been detected in breast cancer tissues, there does not appear to be any significant link between HCMV infection and breast cancer progression (Utrera-Barillas *et al.*, 2013). However, a significant increase in HCMV expression is one of the biomarkers of inflammatory breast cancer (El-Shinawi *et al.*, 2013). In the past decade, HCMV genome and antigens has been detected in various human cancers, including breast cancer, prostate cancer, brain cancer, colon cancer, gastric cancer and salivary gland cancer (Michaelis *et al.*, 2009; Soroceanu & Cobbs, 2011; Jin *et al.*, 2014). HCMV infection is not rare in oesophageal cancer patients, who have undergone stem cell transplantation

or receiving immunosuppressant agents or have human immunodeficiency virus (HIV) infection (Umemoto *et al.*, 2016). In these patients, HCMV has been shown to remain inactive but reactivation of the virus can occur during chemoradiotherapy and may affect the prognosis of the patients, moreover, reactivation of HCMV during chemoradiotherapy is associated with development of cytomegalovirus esophagitis in these patients (Chang *et al.*, 1992; Aizawa *et al.*, 2017). HCMV infection in cancer contributes to BCLAF1 protein degradation which results in reduced *BCLAF1* levels in breast cancer. In HeLa cells, overexpression of *BCLAF1* induces apoptosis (Kasof *et al.*, 1999). Overexpression of *BCLAF1* is suppressed by co-expression of BCL2 and BCL-XL proteins. Anti-apoptotic *BCL2* and *BCL-XL* mRNA levels is low in HeLa cells, hence overexpression of *BCLAF1* was observed in this study.

An investigation of *BCLAF1*-related gene expression in the same cell lines revealed extremely low levels of proapoptotic gene expression (*Caspase-3, Caspase-9* and *BAX*) in the MDA-231 cell line compared to other cell lines. The same was true for the DNA damage repair genes *H2AX*, *BACH1*, *BRCA1* and mostly, the *EXO1*, *ATRIP* genes. Deregulation of proapoptotic genes results in the disruption of the regulation of the balance between proliferation and apoptosis and it is one of the most important traits of malignancy (Marx & Meden, 2001). *BCLAF1* is involved in the induction of Caspase-3, Caspase-9 and BAX apoptotic activity and its deficiency in cells reduces mRNA and protein expression of these genes (Kasof *et al.*, 1999; Lee, Yu, *et al.*, 2012). Thus, reduced transcript levels of *Caspase-3, Caspase-9* and *BAX* were observed in all cell lines but extremely low in the MDA-231 cells. Apoptosis inhibitors such as BCL2 related proteins are known to be over expressed in various tumours, such as epithelial tumours and human B-cell lymphoma and they play an important role in tumourigenesis and progression by inhibiting cell death and prolonging the survival of malignant cells (Tsujimoto *et al.*, 1985; Marx *et al.*, 1997; Yip & Reed, 2008). *BCL2* was observed to be slightly expressed in the MDA-231 cells, over expression of *BCL2* downregulates proapoptotic *BAX* expression (Dagmar Marx & Meden, 2001), hence *BAX* reduced levels observations in the cell lines

BRCA1 is present in low levels or absent in most breast cancers (Garcia *et al.*, 2011) and its low expression predispose women to breast cancer (Welcsh & King, 2001). *EXO1* and *ATRIP* are some of the genes regulated by the BRCA1/BCLAF1 mRNA splicing complex, and the stability and efficient production of their transcripts depends on the activity of the BRCA1/BCLAF1 mRNA

splicing complex (Savage *et al.*, 2014). Furthermore, deregulation or loss of *BRCA1* or *BCLAF1* results in loss of BRCA1/BCLAF1-mediated mRNA splicing activity, hence, ectopic expression of *ATRIP*, BACH1, and EXO1 (Savage *et al.*, 2014). As expected, *ATRIP* and *EXO1* transcript levels were reduced in the all cell lines analysed and extremely low levels in MDA-231 cells that have extremely low expression of *BCLAF1* and *BRCA1*.

Another important feature of *BCLAF1* is its involvement in the regulation of γH2AX-mediated repair of DNA damage associated with either apoptosis or double strand breaks during DNA damage. In irradiated-responsive cells, BCLAF1 translocate to the nuclear envelope and merges with γH2AX, a novel biomarker for DNA double-strand breaks, which in turn stabilizes the Ku70/DNA-PKcs complex involved in facilitating non-homologous end joining double strand break repair (Lee, Yu, *et al.*, 2012). We investigated the expression of *H2AX* and *BAX*-cytoplasmic interacting partner, *Ku70* in several cell lines. In HeLa and MDA-231 cells both *H2AX* and *Ku70* gene expression were strongly repressed compared to the other cell lines. Deregulation of BCLAF1 and H2AX affect Ku70/DNA-PKcs complex stability in the nucleus and reduces the association between Ku70 and DNA-PKcs. Furthermore, it results in reduced Ku70 nucleonic levels whilst enhancing its translocation to the cytoplasm. In the cytosol, the binding of Ku70 to BAX is enhanced, resulting in inactivate BAX. Previous studies have also reported that *BCLAF1* is required to release BAX from the anti-apoptotic Ku70-BAX complex (Lee, Yu, *et al.*, 2012). In MDA-231 cells, *H2AX* transcript levels were extremely low compared to other cell lines, we thus hypothesise that *H2AX* is a *BCLAF1* downstream gene. In contrast, *in vitro* studies have shown that BCL-2 interacts with *BCLAF1* (Kasof *et al.*, 1999) to negatively regulate *BCLAF1* double strand break repair activity by disrupting the Ku70/DNA-PKcs complex (Downs & Jackson, 2004; Lee, Yu, *et al.*, 2012). Moreover, depletion of *BCL-2* enhances the DNA binding activity of the Ku70/DNA-PKcs complex accelerating DNA repair (Wang *et al.*, 2008).

The pathway of the key cell cycle regulator, *P53*, is inactivated in the most human tumours (Herrero *et al.*, 2016). In this study, *P53* which is positively regulated by *BCLAF1* through the CPE-TP53 element in a PKCδ-dependent manner (Liu *et al.*, 2007) was observed to be significantly reduced in all the cancer cell lines. In addition, previous work on siRNA-mediated *BCLAF1* knockdown studies indicated that *BCLAF1* deficiency significantly attenuates *P53* mRNA levels, leading to a reduction of *P53*-dependent apoptosis activity (Liu *et al.*, 2007).

Depending on the severity of DNA damage, *P53* determines the cell fate through activation of different pathways (Luo *et al.*, 2017). The cyclin-dependent kinase inhibitor *P2*1 and pro-apoptotic genes such as *BAX*, are some of the *P53* target genes (Luo *et al.*, 2017). Deregulation of *P21* results in cell cycle deregulation and uncontrolled cell growth (Elbendary *et al.*, 1996). The over-expression of *P21* in oesophageal cancer cell lines; KYSE30 and WHCO1 results in *P21*-dependent cell cycle arrest at G1 phase.

Cell cycle analysis by flow cytometry indicated that MDA-231 cells have a distinct profile, with more cells in the S-phase and less in the G1 phase and more apoptotic cells compared to the other cell lines. Although *BCLAF1*- and *P53*-mediated apoptosis pathways are deregulated in the MDA-231 cells, due to the low levels expressions, the presence of excessive apoptotic cells could be due to activation of extrinsic apoptotic pathway, initiated by the interaction of exposed cell surface death receptors, belonging to the superfamily of tumour necrosis factor receptors (TNFR) (Guicciardi & Gores, 2009). The downstream effect of the initiation of the extrinsic apoptotic pathway induces the activation of initiator pro-caspase-8 and/or Caspase-10 resulting in the formation of the death-inducing signalling complex (DISC), which triggers the processing of the downstream effectors such as Caspases-3, Caspase-6 and Caspase-7 whose activation leads to the cleavage of critical cellular substrates, inducing cell death (Degterev *et al.*, 2003).

Taken together, the data suggests that *BCLAF1* expression in different cell types is influenced by various factors, depending on the cause(s) and development of cancer. This study suggests that deregulation and differential expression of *BCLAF1* and of *BCLAF1*-associated genes in the cell lines could result in dysfunctional regulation of signalling pathways such as apoptosis, DNA damage repair and cell cycle which play critical roles in development and progression of cancer. The downstream effects of *BCLAF1* knockdown and its molecular role in tumourigenesis is presented in chapter 3.

CHAPTER 3

The downstream effects after *BCLAF1* knockdown and knockout

3.1 Introduction

The use of RNA interference (RNAi) technology is a very potent tool in the modification of gene expression. Furthermore, it is used for investigating the stimulatory or suppressive role of a given gene in biological pathways, to validate gene function, its relation to the disease phenotypes and as a tool for gene therapy in cancer and other genetic diseases (Guo *et al.*, 2013; Kaufmann *et al.*, 2013; Barrangou *et al.*, 2015). Several studies using RNAi technology have suggested a functional role for *BCLAF1* functional in several cancer signalling pathways, consistent with the hallmarks of cancer (McPherson *et al.*, 2009; Sarras *et al.*, 2010; Lee, Yu, *et al.*, 2012; Sarras, 2012; Zhou *et al.*, 2014; Shao *et al.*, 2016; Vohhodina *et al.*, 2017).

BCLAF1 was originally identified in a yeast two-hybrid screen of proteins that interact with E1B 19K. E1B-19k is one of the two proteins encoded by the E1B gene of the adenovirus, which blocks *P53*-independent apoptosis mechanism in adenovirus-infected cells (White & Cipriani, 1990; Lowe & Ruley, 1993; White, 2001). Subsequent studies indicated *BCLAF1* as a protein-protein interacting partner with BCL2 family members and its overexpression induced apoptosis in HeLa cells and suppresses transformation by adenovirus E1A and E1B-19K proteins or mutant *P53* (Kasof *et al.*, 1999). McPherson and his colleagues used *in-vivo* knockdown studies of *BCLAF1* to confirm the role of *BCLAF1* in the cell death signalling pathway, and in proper lung development, T-cell activation and immune system homeostasis in *BCLAF1* knockout mice (McPherson *et al.*, 2009). Furthermore, *BCLAF1* deficiency by siRNA caused cell resistance to ceramide-induced apoptosis, whereas overexpression of *BCLAF1* induced the upregulation of *P53* and *BAX* levels, but down-regulating *MDM2* levels (Sarras *et al.*, 2010). Studies using siRNA-mediated knockdown identified further roles for *BCLAF1* in other process such DNA damage repair, mRNA splicing and stabilization of transcripts, suggesting that *BCLAF1* is a tumour suppressor (Lee, Yu, *et al.*, 2012; Savage *et al.*, 2014).

The work in this chapter was designed provide some insight into the contribution of *BCLAF1* in tumourigenesis by examining the effects of *BCLAF1* deficiency in signalling pathways such as apoptosis, DDR and cell cycle.

3.2 Results

3.2.1 siRNA-mediated *BCLAF1* knockdown

Transformed human lung fibroblasts CT1 cells were used as a model to study and identify the effects of downstream effects of *BCLAF1* knockdown and its contribution in tumorigenesis. CT1 cells were treated with *BCLAF1* siRNA. *BCLAF1* knockdown was validated at both the protein and RNA level 24 hours post transfection (**figure 3.1**).

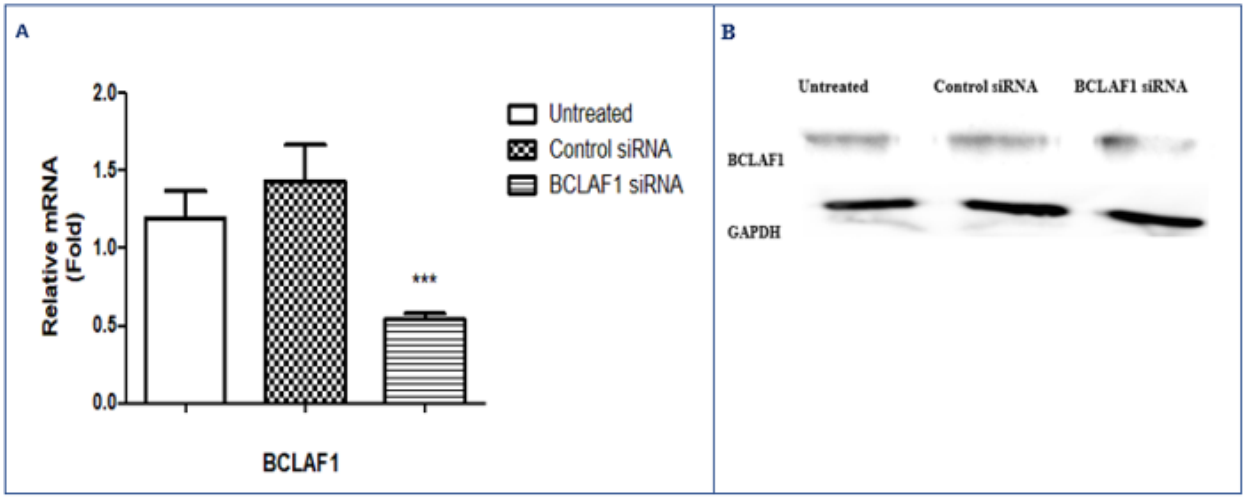

Figure 3.1. siRNA-mediated *BCLAF1* knockdown. CT-1 cells were treated with either scrambled siRNA as negative control or *BCLAF1* siRNA. RNA and protein extracts were harvested from the cells 24 hours post-transfection. **A**); *BCLAF1* mRNA was quantitated using specific primers as indicated in Material and Methods in Table 5.5. **B**); Protein extracts were electrophoresed on a 10% polyacrylamide SDS gel, transferred to nitrocellulose membranes and probed for BCLAF1 protein using an anti-BCLAF1 antibody. The gels were stripped and probed with an anti-GAPDH antibody to serve as a loading control.

Analysis of *BCLAF1* mRNA by RT-PCR analysis in control siRNA and *BCLAF1* siRNA treated cells (**Figure 3.1A**) showed that the siRNA successfully knocked down *BCLAF1* mRNA levels by at least 50% compared to the untreated or control non-specific scrambled siRNA. Western blot analysis confirmed that the BCLAF1 protein level was also significantly reduced in the *BCLAF1* siRNA treated cells (**Figure 3.1B**). Previous *in vitro* studies on *BCLAF1* knockdown achieved a 60-30% *BCLAF1* knockdown (Lee, Yu, *et al.*, 2012; Savage *et al.*, 2014).

3.2.2 The effect of *BCLAF1* on cellular gene expression

To assess whether the expression of genes downstream of *BCLAF1* was altered by the targeted disruption of *BCLAF1* expression, RNA and protein extracts from either non-specific scrambled control siRNA, *BCLAF1* siRNA and untreated cells were collected 24 hours post-transfection. RT-

PCR and western blot analyses were carried out to determine the effects of *BCLAF1* deficiency on proapoptotic, anti-apoptotic, DDR gene expression and cell cycle regulators.

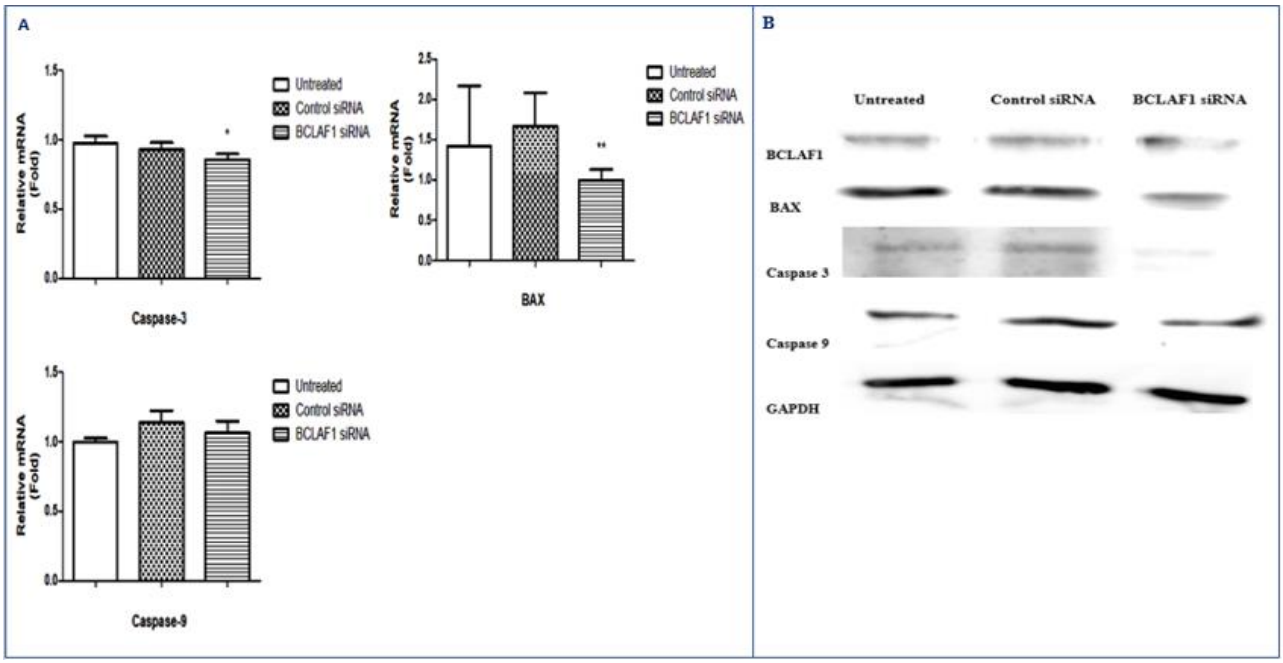

Figure 3.2. The effect of BCLAF1 knockdown on proapoptotic gene expression. RNA and protein extracts were prepared from CT1 cells 24 hours post-transfection with either control siRNA or BCLAF1 siRNA as described in Material and Methods. A); RT-PCR analysis of the proapoptotic genes BAX, Caspase-3 and Caspase-9 mRNA expression was quantitated using specific primers as indicated in Material in Table 5.5. B); Western blot analysis of BAX, Caspase-3 and Caspase-9 proteins in BCLAF1 siRNA-treated cells. GAPDH was used as a loading control. This experiment was done in triplicates.

The data in (**Figure 3.2A**) and (**Figure 3.2B**) showed that knockdown of *BCLAF1* expression had a significant effect on the expression of the apoptotic genes and downregulated *BAX* expression both at RNA and protein level, Caspase-3 protein levels were reduced.

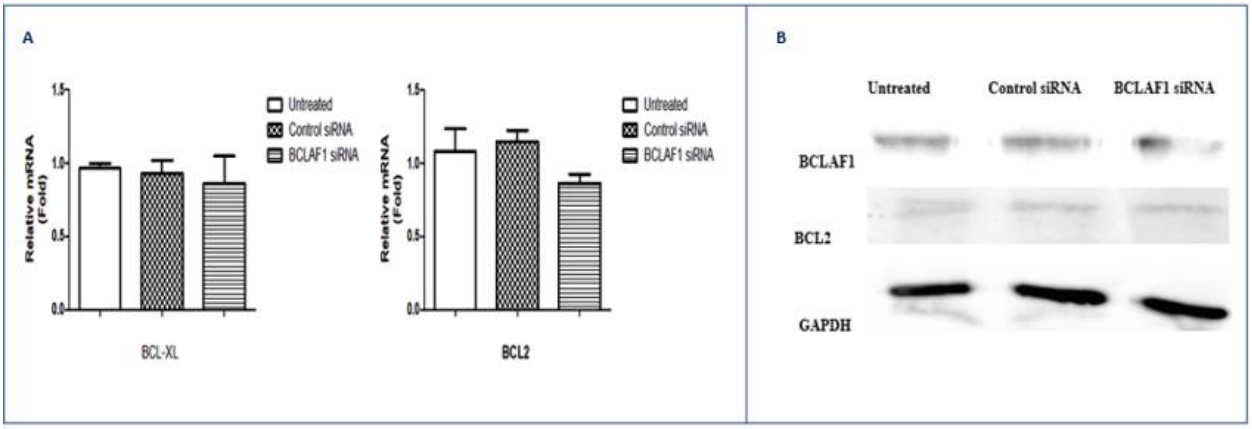

Figure 3.3. The effect of *BCLAF1* knockdown on anti-apoptotic gene expression. RNA and protein extracts were prepared from CT1 cells 24 hours post-transfection with either control siRNA or BCLAF1 siRNA as described in Material and Methods. A); RT-PCR analysis of the antiapoptotic genes BCL2 and BCL-XL. B); Western blot analysis of BCL2 protein in BCLAF1 siRNA-treated cells. GAPDH was used as a loading control. This experiment was done in triplicates. BCL-XL antibody suitable for western blot was not commercially available.

Analysis of antiapoptotic mRNA expression by RT-PCR analysis showed no significant effect in the *BCL2* expression both at RNA and protein levels as well as *BCL-XL* mRNA expression (**Figure 3.3**).

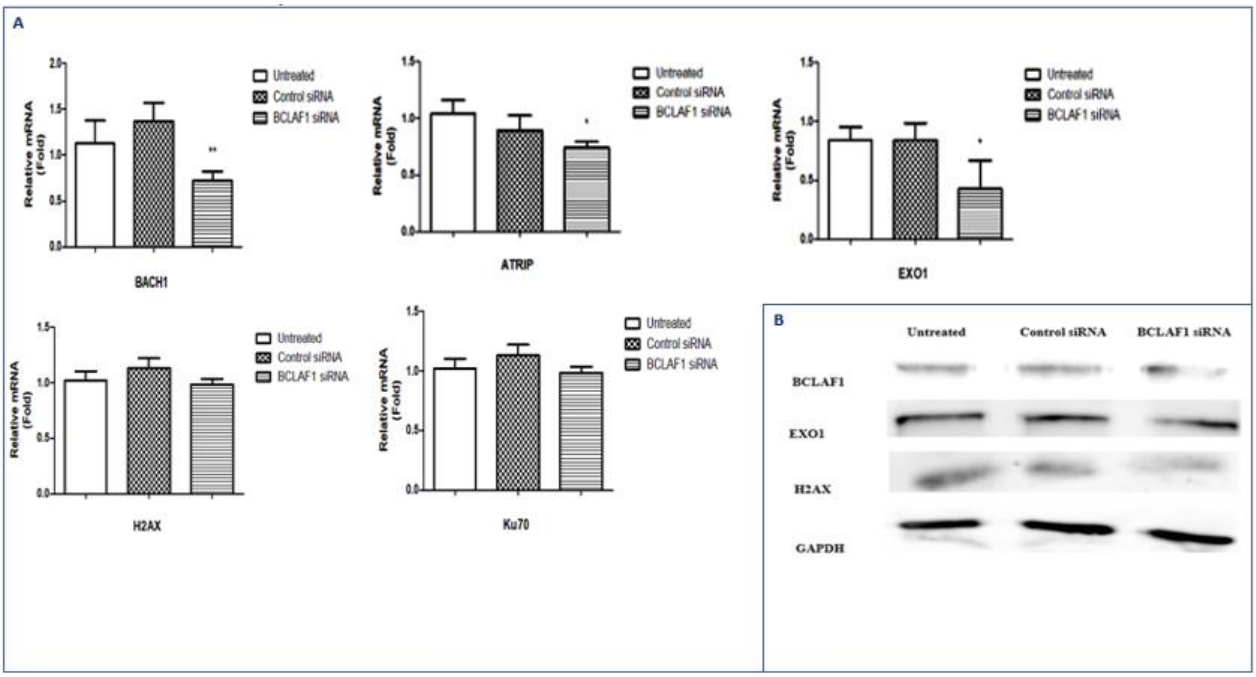

Figure 3.4. The effect of BCLAF1 knockdown on DDR gene expression. RNA and protein extracts were prepared from CT1 cells 24 hours post-transfection with either control siRNA or BCLAF1 siRNA as described in Material and Methods. A); RT-PCR analysis of the DDR genes using specific primers as indicated in Material and Methods in Table 5.5. B); Western blot analysis of DDR protein in BCLAF1 siRNA-treated cells. GAPDH was used as a loading control. This experiment was done in triplicates. ATRIP, BACH1 and Ku70 antibodies suitable for western blot were not commercially available.

Depletion of *BCLAF1* results in reduced mRNA levels of *EXO1*, *ATRIP* and *BACH1* (**Figure 3.4A**). *BCLAF1* knockdown attenuated EXO1 and H2AX protein levels (**Figure 3.4B**).

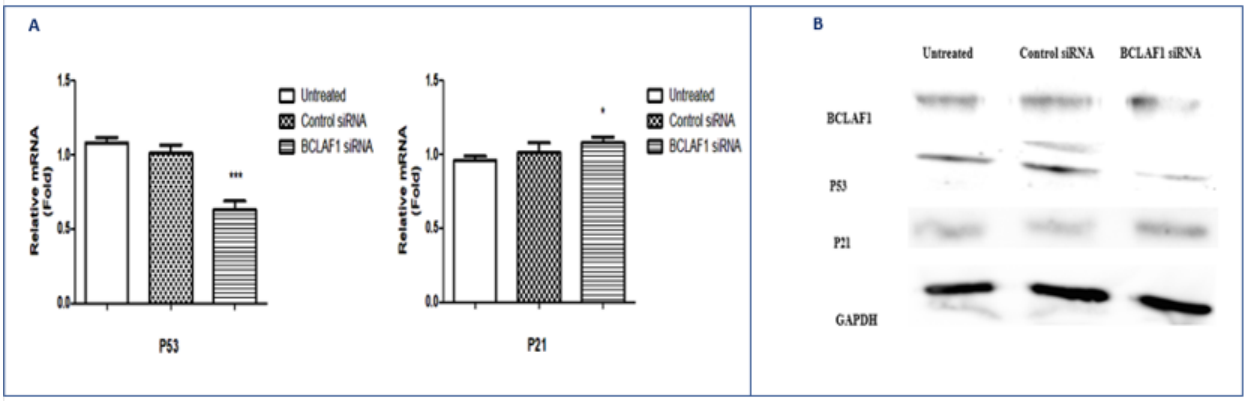

Figure 3.5. *BCLAF1* knockdown effects on cell cycle regulators. RNA and protein extracts were prepared from CT1 cells 24 hours post-transfection with either control siRNA or *BCLAF1* siRNA as described in Material and Methods. **A**); RT-PCR analysis of the cell cycle regulator P53 and P21. **B**); Western blot analysis of P53 and P21 protein in *BCLAF1* siRNA-treated cells. GAPDH was used as a loading control.

Knocking down *BCLAF1* attenuated *P53* expression both at RNA and protein levels, and slightly upregulated *P21* mRNA and protein levels (**Figure 3.5**).

3.2.3 *BCLAF1* knockdown effects on cell cycle and apoptosis

BCLAF1 is previously reported to be involved in *P53* transcription activation and in the γH2AX-mediated regulation of *P53*/*P21* expression and cell cycle progression (H. Liu et al., 2007; Y. Y. Lee et al., 2012), meanwhile, *BCLAF1* knockdown altered the expression of cell cycle regulators by downregulating *P53* expression and upregulating *P21*. The effects of *BCLAF1* knockdown on cell cycle progression was determine by flow cytometric analysis CT1 cell transfected with either control siRNA or *BCLAF1* siRNA and the untreated cells were harvested 24 hours post-transfection.

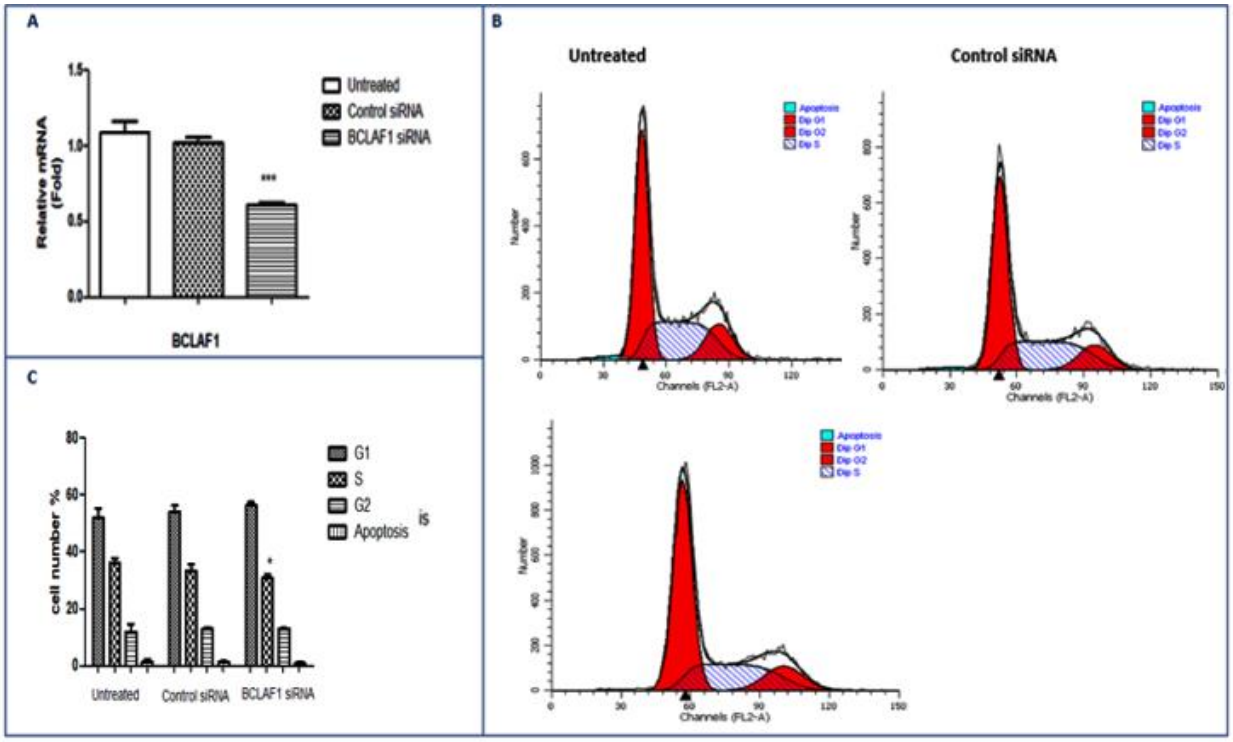

Figure 3.6. ***BCLAF1*** **knockdown on the cell cycle progression.** CT1 cells were transfected with either control siRNA or *BCLAF1* siRNA. Cells were stained with PI and analysed using a FACScan-cell sorter. Each sample was analysed in triplicate and 20 000 events were captured. **A**); *BCLAF1* knockdown was validated using specific primers as indicated in Material and Methods in Table 5.5. **B**); *BCLAF1* knockdown effects on cell cycle flow. **C**); The bar graphs showed the relative quantity of G1, S, G2 and apoptosis population in the CT1 *BCLAF1* siRNA transfected cells compared to the untreated and control siRNA.

Analysis of *BCLAF1* mRNA by RT-PCR analysis in control siRNA and *BCLAF1* siRNA treated cells showed that the siRNA successfully knockdown *BCLAF1* mRNA levels by more than 50% compared to the untreated or control non-specific scrambled siRNA (**Figure 3.6A**). *BCLAF1* knockdown reduced levels of apoptotic cells and a slightly accumulation of cells in the G1 phase and a statistically significant re-entry to S phase (**Figure 3.6B-C**).

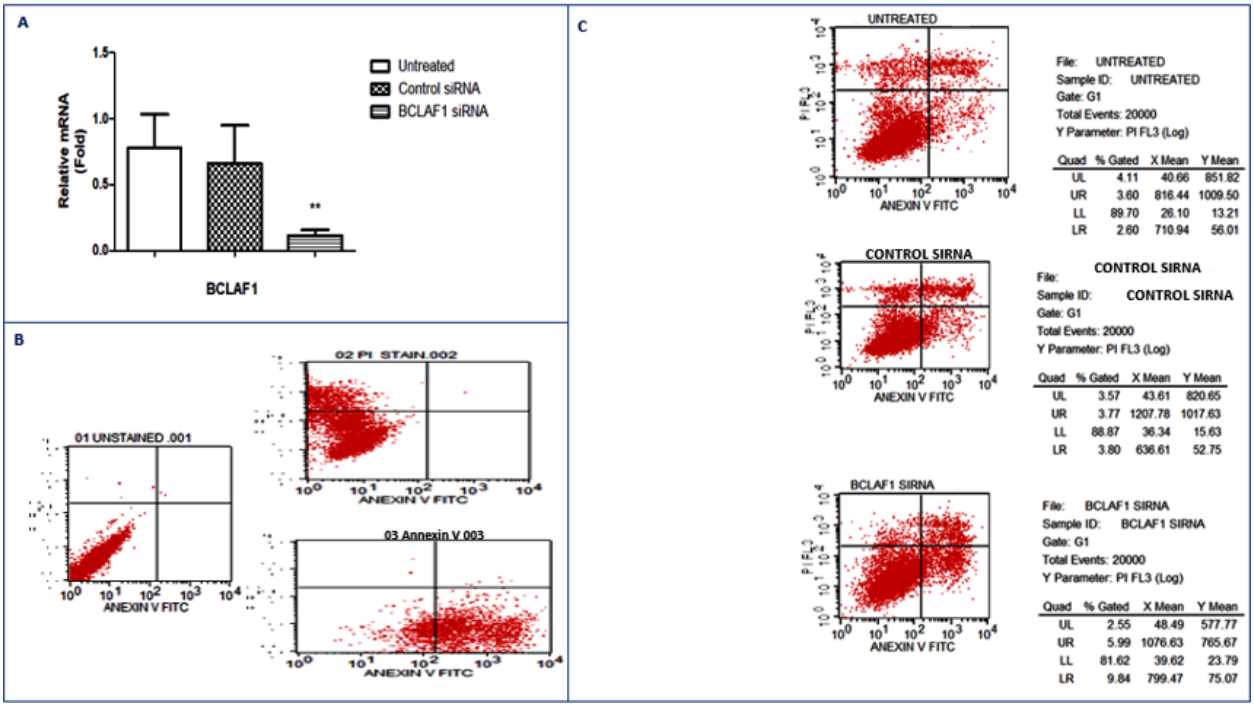

Figure 3.7. Effects of *BCLAF1* knockdown on apoptosis. CT1 cells were transfected with either control siRNA or BCLAF1 siRNA. Cells were stained with PI and Annexin V and analysed using a FACScan-cell sorter. Each sample was analysed in triplicate and 20 000 events were captured. **A**); 24 hours post transfection, *BCLAF1* knockdown was validated using specific primers as indicated in Material and Methods in Table 5.5. **B**); Single stain positive controls; CT1 cells were either stained with PI only, Annexin V stain only or unstained. **C**); FITC Annexin V/PI double staining of cells.

Analysis of *BCLAF1* knockdown mRNA by RT-PCR analysis in control siRNA and *BCLAF1* siRNA treated cells showed that the siRNA successfully knocked down *BCLAF1* mRNA levels by about 75% compared to the untreated or control non-specific scrambled siRNA (**Figure 3.7A**). Viable cells are FITC Annexin V and PI negative are in the lower left quadrant; cells that are in early apoptosis are FITC Annexin V positive and PI negative, in the lower right quadrant; and cells that are in late apoptosis or already dead are both FITC Annexin V and PI positive in the upper right quadrant (**Figure 3.7B**). *BCLAF1* knockdown reduced dead cells population in *BCLAF1* siRNA treated cells with a population of 2.55% compared to untreated and control siRNA cells with a dead population of 4.11% and 3.57% respectively (**Figure 3.7C**).

3.2.4 The effect of *BCLAF1* on DNA damage and Repair

In order to determine whether *BCLAF1* promotes resistance to DNA damage, we examined the effect of *BCLAF1* depletion on cellular survival after UV induced DNA damage. The optimum ultraviolet (UV) dose based on cell survival was determined 24 hours post UV induced DNA

damage. UV exposure dosages were 0J/M^2, 40J/M^2, 200J/M^2 and 500J/M^2. DNA was extracted electrophoresed on a 1% agarose gel to visualise DNA fragmentation.

3.2.4.1 UV radiation induce DNA damage and cell survival

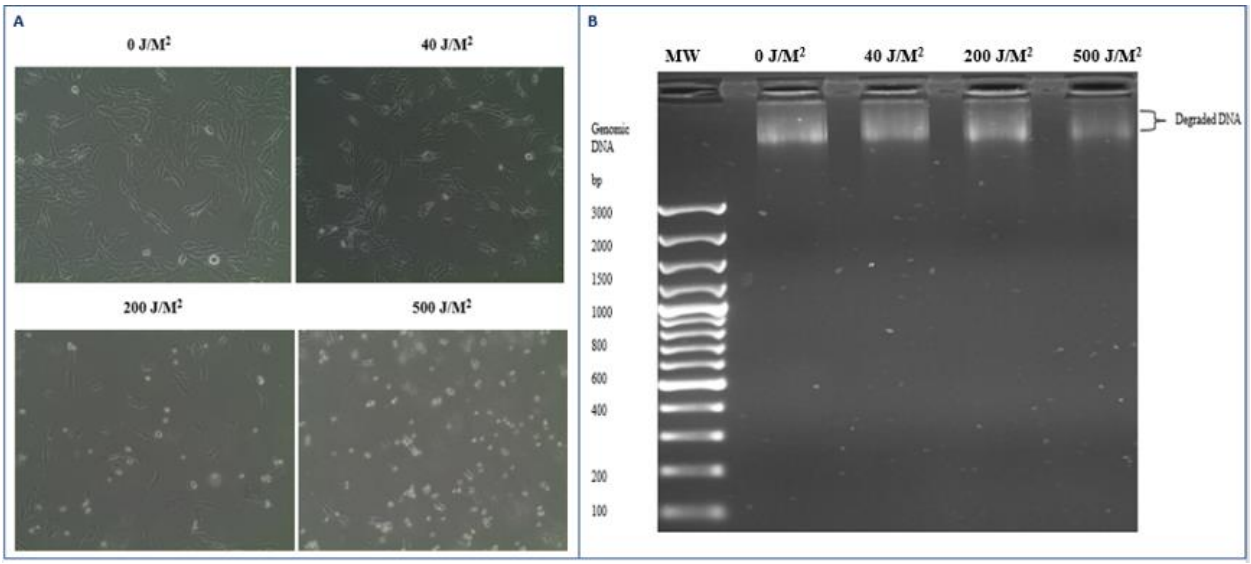

Figure 3.8. UV induced DNA damage and cell survival. 2.0x10^5 CT1 cells/well were seeded on 6-well plates and left to attach and grow overnight. Cells were treated with UV light at the indicated doses and let to recover for 24 hours. **A**); Cell morphology changes were observed by microscopy in cells. **B**); DNA electrophoretic profiles analysed on a 1% agarose gel. MW, DNA size marker. Each experiment was performed three independent times.

The results in (**Figure 3.8A**) showed the presence of morphological changes such as cell shrinkage in cells exposed at 200 and 500 J/M^2, 80-90% of the population died compared with unexposed cells, while cells exposed to 40J/M^2 morphologies remained the same and cells were able to recover and survived 24 hours post UV treatment. DNA degradation was observed in all the cells exposed at 40,200 and 500 J/M^2 doses (**Figure 3.8B**). 40 J/M^2 was used as the optimum dose in further analysis.

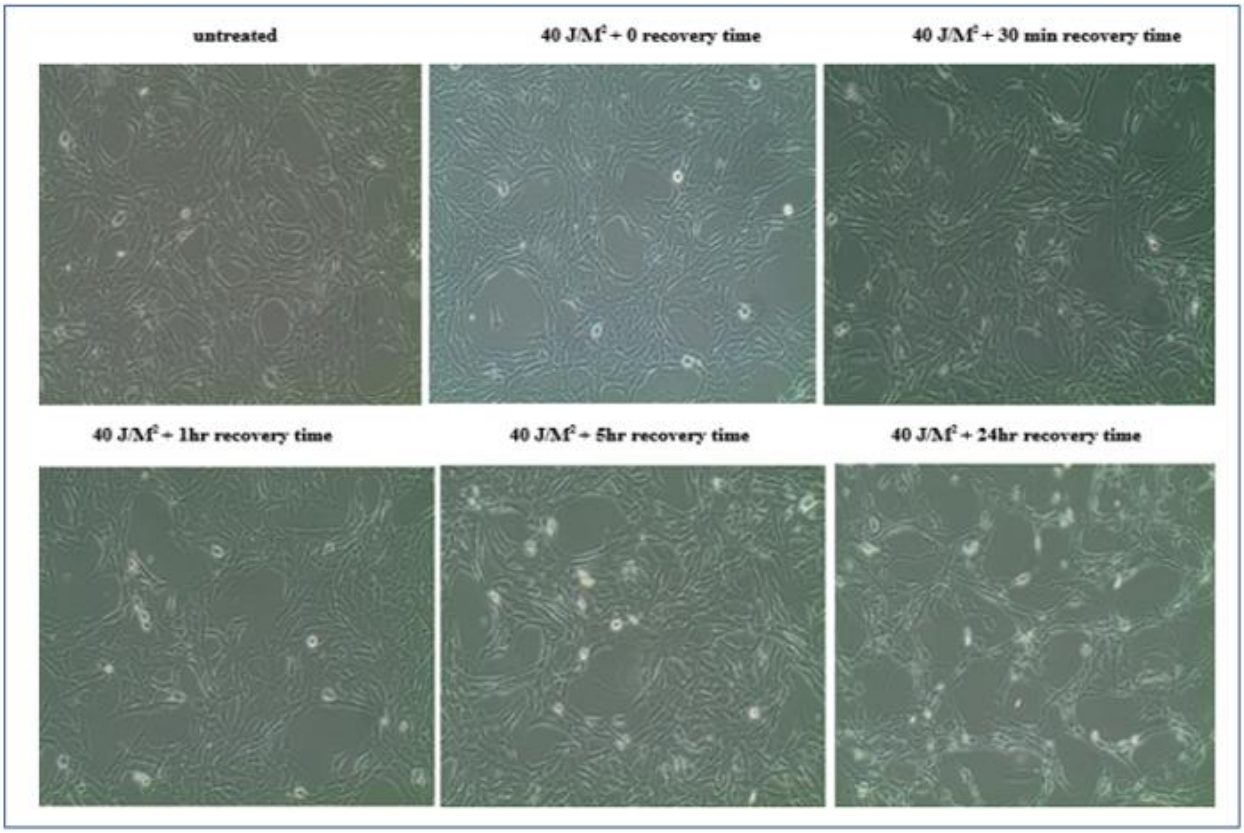

Figure 3.9. Recovery of cells after UV irradiation. CT1 cells were treated with 40 J/M^2 UV light and left to recover for either 0.5, 1, 5 or 24 hours. At each time point, the number of surviving cells were observed under a microscope and compared to the untreated control. Cell viability decreased dependent on recovery time point.

The results in (**Figure 3.9**) showed that depending on the recovery time given and if the extent of DNA damage exceeds the capacity of repair mechanisms, cells go through apoptosis. About 20-30% of the population of cells with 24 hours recovery time died compared with untreated cells.

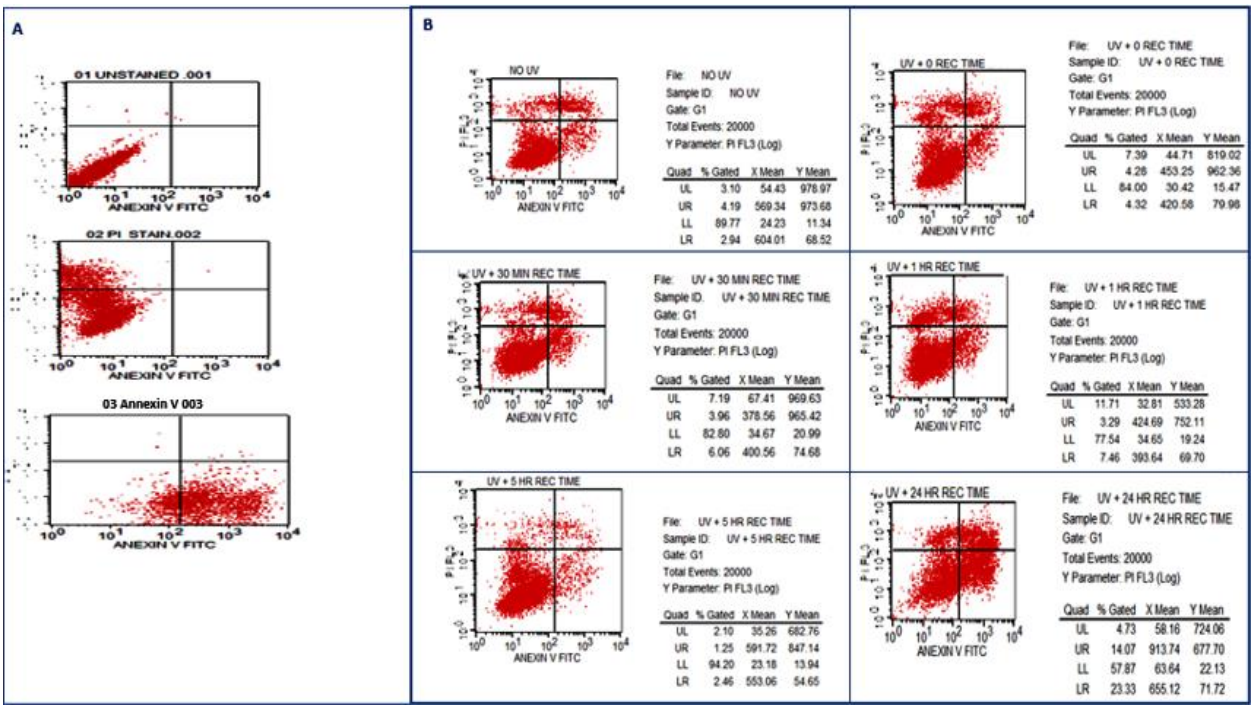

Figure 3.10 The effect of UV radiation on the cell survival and apoptosis. CT1 cells were treated with 40 J/M^2 UV light and left to recover for either 0.5, 1, 5 or 24 hours. At each time point cells were stained with PI and Annexin V and analysed using a FACScan-cell sorter. Each sample was conducted in triplicate and 20 000 events were captured. **A**); Single stain positive controls; CT1 cells were either stained with PI only, Annexin V stain only or unstained. **B**); FITC Annexin V/PI double staining of cells.

The data in (**Figure 3.10**) showed that untreated cells (No UV) and UV treated cells with 0 hours recovery time (UV+0 recovery time) were primarily FITC Annexin V and PI negative i.e. more viable cells. Early apoptotic cell population i.e. Annexin V-positive but PI-negative cells increased in a recovery time-dependent manner (cells with 0.5, 1-hour recovery time). Cells that are left for 24 hours to recover had a high population of the early apoptotic cells so as dead cells population with 23.33% and 14.07% respectively, and less viable cells population compared to untreated cells.

3.2.4.2 *BCLAF1* knockdown effects in DDR

To examine the ability of *BCLAF1*-depleted cells to recruit γ-H2AX to damaged DNA sites for effective DNA repair, a time-course and cell survival test was carried out to determine the optimal time-course selection dependent on the survival of cells and sustained cell morphology post UV exposure see (**Figure 3.9**) and (**Figure 3.10**). After 24 hours, *BCLAF1* siRNA-transfection cells and untreated cells were exposed to 40J/M^2 UV dose for immunofluorescence analysis. *BCLAF1* knockdown validation (**Figure 3.11**).

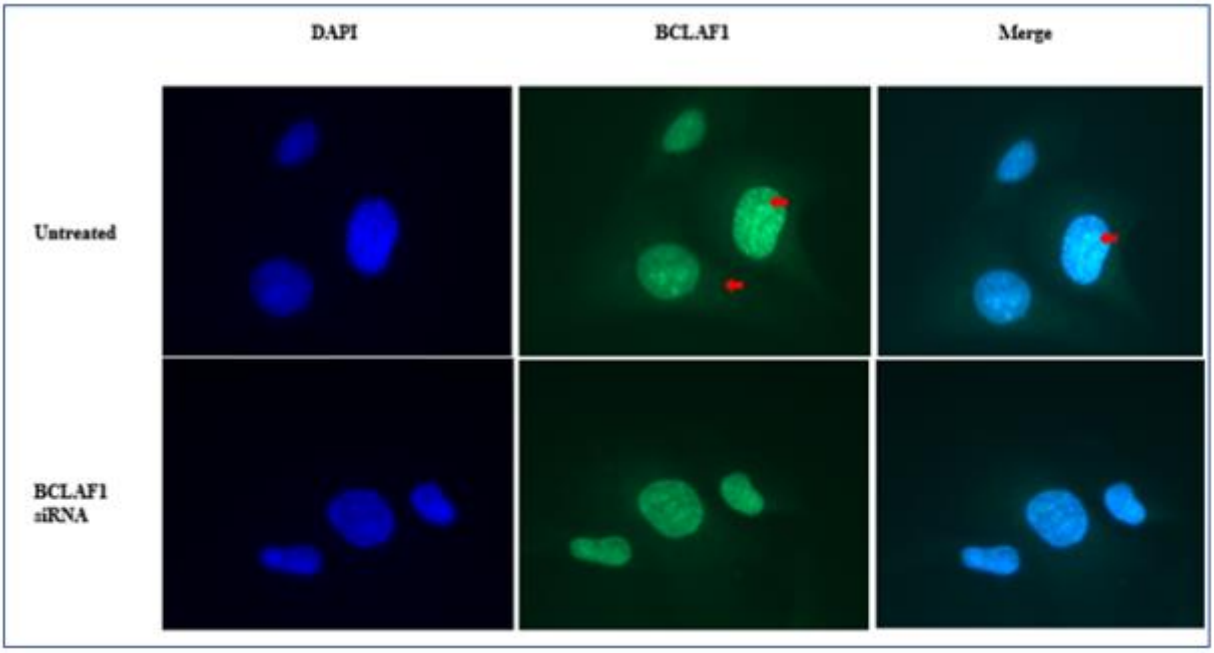

Figure 3.11 *BCLAF1* knockdown validation. Representative immunofluorescent staining of *BCLAF1*. $2.0x10^5$ CT1 cells/well were seeded on cover slips in 6-well plates overnight. 24 hours post *BCLAF1* siRNA transfection as described in Materials and Methods, cells were fixed and stained for *BCLAF1*. Left: nuclear staining with DAPI; Middle: cell nucleus as well as cytoplasm incubated with anti-BCLAF1 antibody; Right: merged image of *BCLAF1* and DAPI staining. The red arrows indicate the localization of *BCLAF1*. Images were obtained at 400x.

Analysis of *BCLAF1* expression in untreated and *BCLAF1* siRNA treated cells showed a decrease in the cytoplasmic and nuclear *BCLAF1* expression in *BCLAF1* siRNA treated cells compared to untreated cells.

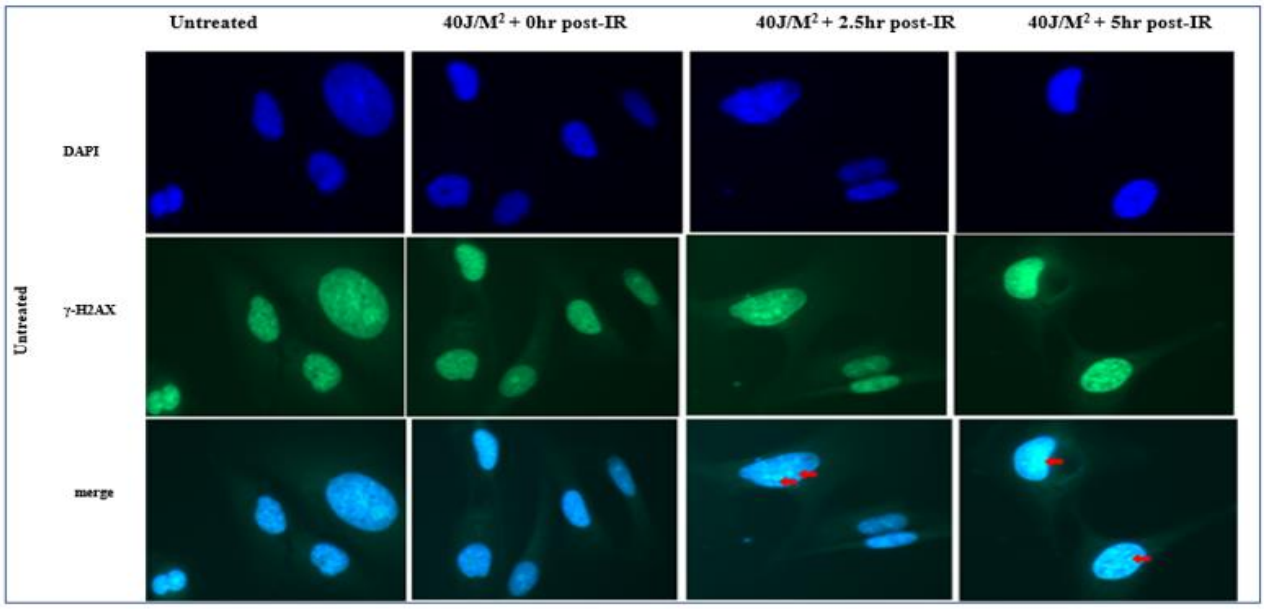

Figure 3.12 Damaged DNA repair in CT1 cells post UV exposure. Representative immunofluorescent staining of γ-H2AX marked DNA damage in CT1 no siRNA-40 J/M^2 UV-treated cells and 0, 2.5 and 5 hours recovery time. Cells were fixed and stained for γ-H2AX as described in Materials and Methods. Top row: nuclear staining with DAPI; Middle: cell nucleus as well as cytoplasm incubated with anti-γH2AX antibody; bottom row: merged image of γH2AX and DAPI staining. The red arrows indicate the localization of γ-H2AX-damaged DNA marked sites. Images were obtained at 400x. Each time point was performed in triplicate.

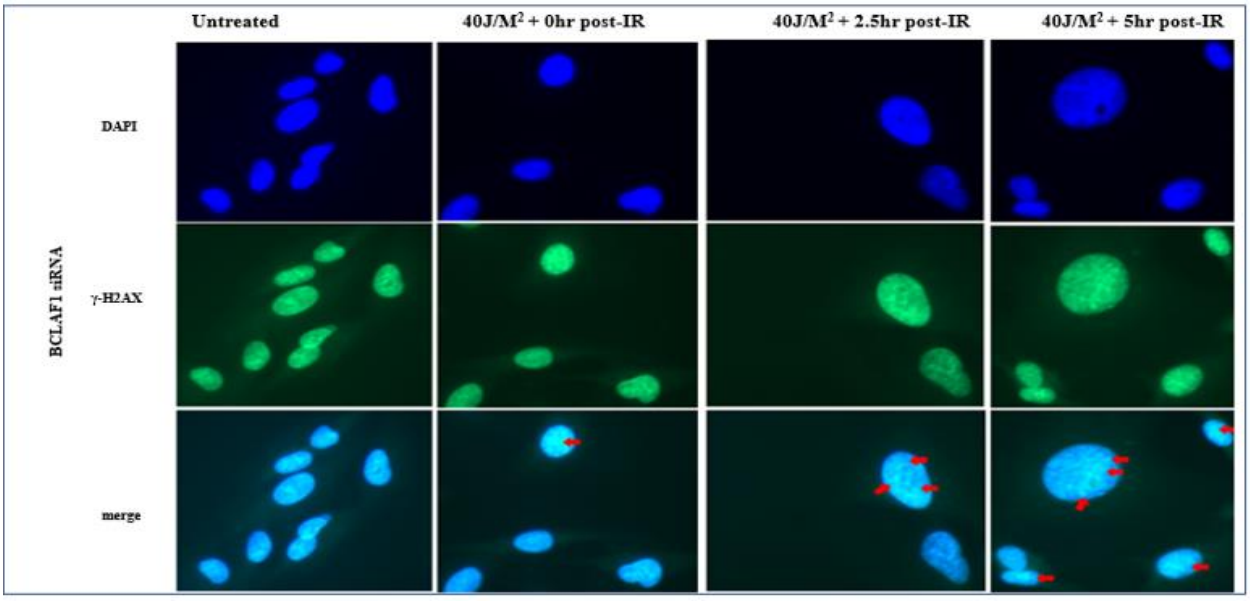

Figure 3.13 *BCLAF1* mediates Resistance to DNA damage and is required for efficient DNA repair. Representative immunofluorescent staining of γ-H2AX marked DNA damage in CT1 siRNA-mediated *BCLAF1* depleted 40J/M^2 UV-treated cells and 0, 2.5 and 5 hours recovery time. Cells were fixed and stained for γ-H2AX as described in Materials and Methods. Top row: nuclear staining with DAPI; Middle: cell nucleus as well as cytoplasm incubated with anti-γH2AX antibody; Bottom row: merged image of γH2AX and DAPI staining. The red arrows indicate the localization of γ-H2AX-damaged DNA marked sites. Images were obtained at 400x. Each time point was performed in triplicate.

Results in (**Figure 3.12** and **Figure 3.13**) showed that *BCLAF1*-depleted CT1 cells showed a significant defect in their ability to resolve γ-H2AX-marked damaged DNA sites 5 hours after UV treatment. These cells displayed an increase in damaged DNA sites in comparison to untreated cells, indicating that loss of *BCLAF1* results in reduced γH2AX repair activities and an increased in defective DNA repair and genomic instability following DNA damage.

3.2.5 CRISPR/Cas9-mediated *BCLAF1* knockout

In order to get complete knockout of *BCLAF1* expression, CRISPR/Cas9-mediated *BCLAF1* knockout was carried out. Three samples were set up, untreated, Cas9 only control and co-transfection with Cas9 nuclease expression plasmid and two distinct *BCLAF1* guide RNAs (gRNAs) (**Table 3.1**). The two gRNAs targeted *BCLAF1* exon 4 and exon 7, thereby silencing *BCLAF1* and establishing a *BCLAF1* knockout cell line. Enrichment of the mKate-Cas9 expressing cells was done by fluorescence-activated cell sorting (FACS) analysis 72 hours post-transfection, to increase the frequencies of cells with Cas9-induced gene engineering events. Two months post clone selection and expansion, RNA and protein extracts were harvested from cells with either mKate2-Cas9 nuclease expressing plasmid only or mKate2-Cas9 nuclease expression plasmid and *BCLAF1* gRNAs and untreated cells for CRISPR/Cas9-mediated *BCLAF1* mutational analysis and examine the downstream effect of *BCLAF1* deletion in tumourigenesis.

3.2.5.1 CRISPR/Cas 9 transfection on CT1 cell line

Table 3.1 *BCLAF1* knockout sample set up. Three samples were prepared; untreated control sample (cell viability), Cas9-expressing plasmid only control sample (Negative control) and Cas9-expressing plasmid+tracrRNA+BCLAF1gRNAs treated sample (BCLAF1 knockout).

Sample	Purpose
Untreated	BCLAF1 wild type
Cas 9 treated	Negative control
Treated (BCLAF1 crRNA)	BCLAF1 knockout

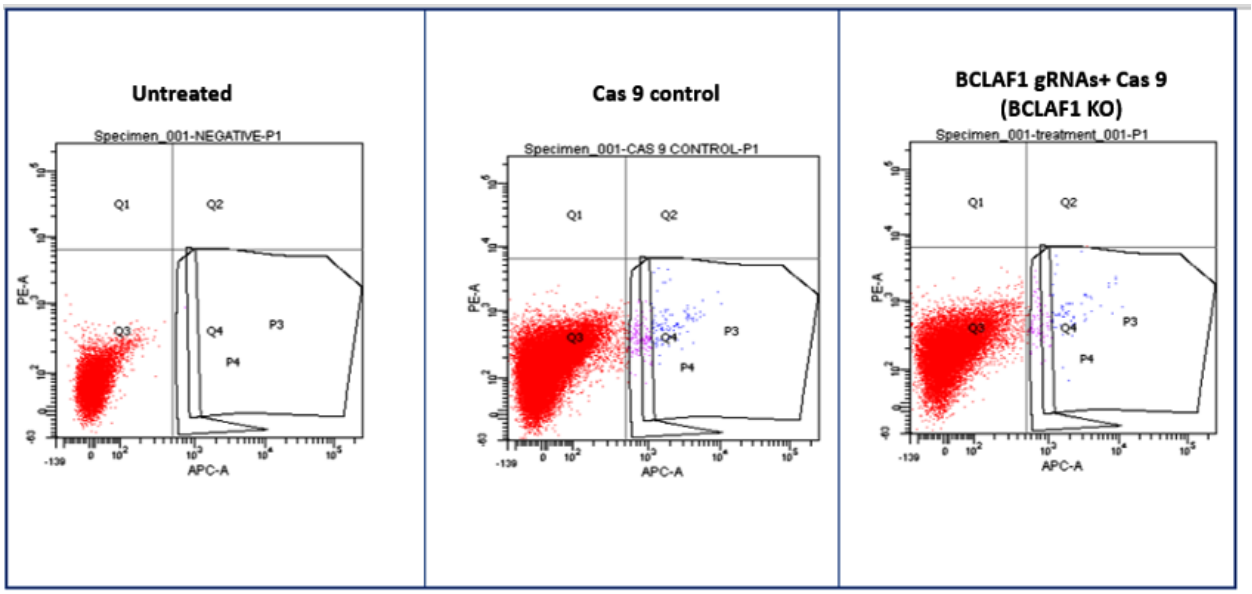

Figure 3.14 FACS plots analysis of CT1 cells post-transfection. $1x10^5$ cells were seeded on 24-well plates overnight, 72 hours post-transfection with either Cas9 plasmid only or Cas9 plasmid+ tracrRNA + two *BCLAF1* gRNAs and the untreated cells were FAC-sorted using FACSDiva Version 6.1.3, depending on the expression of the mKate2 chromophore of an excitation maximum of 588 nm and emission maximum of 633 nm. mKate2-expressing cells of Cas9 only and CRISPR/Cas9-mediated *BCLAF1* knockout cells were drop-pooled in a 6-well plate.

Data in (**Figure 3.14**) showed that both Cas9-expressing control and *BCLAF1* knockout cells showed a positive population of mKate2-Cas9 expressing cells compared to untreated cells which showed no frequency of mKate2-Cas9 positive cells in quantrant 4 (Q4).

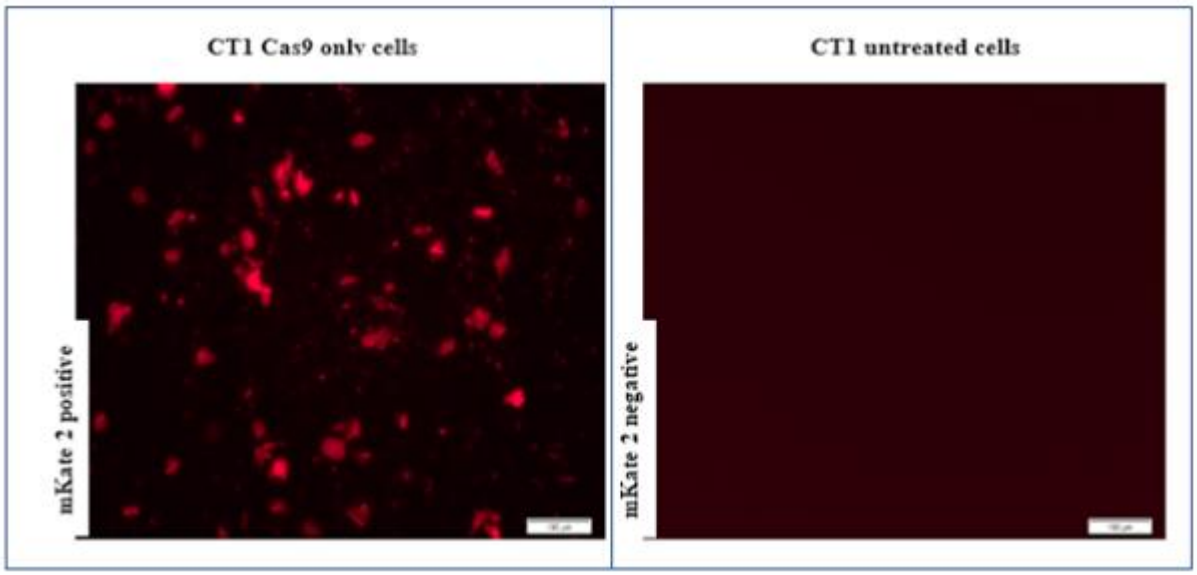

Figure 3.15 Expression of mKate2 in CT1- Cas9 only treated cells. Two weeks post clone selection and expansion, negative control Cas9 only cells and untreated cells were observed and photographed under fluorescence microscope for mKate2 expression, a monomeric far-red fluorescent protein.

CT1-Cas9 cells expressed mKate2, substantial decrease in mKate2 expression was observed three-four weeks later this is associated with increasing passage number. *BCLAF1* knockout treated cells were not used for imaging in avoidance of contamination (**Figure 3.15**).

3.2.5.2 *BCLAF1* knockout validation

In order to assess whether the knockout of *BCLAF1* has the same effects on *BCLAF1* downstream genes expression and in tumourigenesis as the knockdown of *BCLAF1*. Western blot analysis was carried out to validate the knockout of *BCLAF1*.

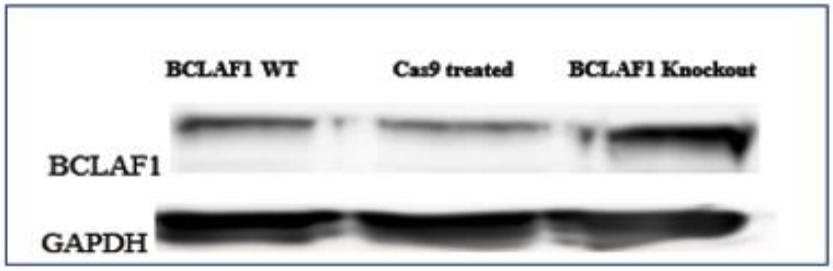

Figure 3.16 CRISPR/Cas9-mediated *BCLAF1* knockout validation. Protein extracts were prepared from CT1 cells with either Cas9 nuclease expressing plasmid only, *BCLAF1* knockout and untreated cells as described in Material and Methods. GAPDH was used as a loading control.

Analysis of BCLAF1 protein expression in untransfected cells, Cas9 only transfected and *BCLAF1* knockout cells by western blot analysis (**Figure 3.16**) showed that CRISPR/Cas9-mediated *BCLAF1* knockout upregulated *BCLAF1* protein levels.

3.2.5.3 CRISPR/Cas9-mediated *BCLAF1* knockout mutational analysis

To clarify the absence of *BCLAF1* deletion but over-expression of *BCLAF1* in CRISPR/Cas9-mediated *BCLAF1* knockout, mutational analysis of *BCLAF1* wild type (untreated cells) and *BCLAF1* knockout cells was done. With the knowledge of where the *BCLAF1* gRNAs targeted (exon 4 and exon 7), two PCR primer sets were designed, (primer set 1 and primer set 2) each set comprised of a forward and a reverse primer targeting regions flanking the targeted regions, upstream and downstream of exon 4 and exon 7 respectively (**Figure 3.18**). RNA extracts from either *BCLAF1* wild type and *BCLAF1* knockout cells was used to synthesize cDNA and then used as a template to amplify the *BCLAF1* targeted regions by polymerase chain reaction (PCR). The PCR products were characterized by 1% agarose gel electrophoresis (**Figure 3.19**).

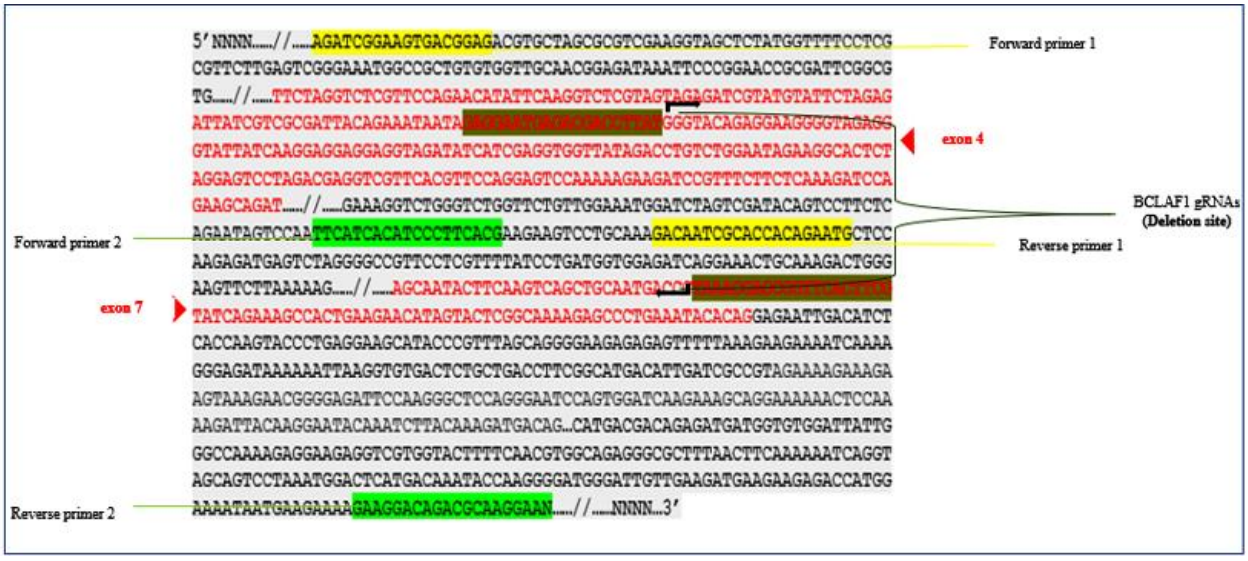

Figure 3.17 PCR primer design. Two sets of primers were designed *in silico*, primer set 1 (highlighted in yellow) and primer set 2 (highlighted in green) targeting exon 4 and exon 7 (red font) respectively. *BCLAF1* gRNAs highlighted in olive green. Each primer set comprised of a forward and a reverse primer, upstream and downstream targeted regions.

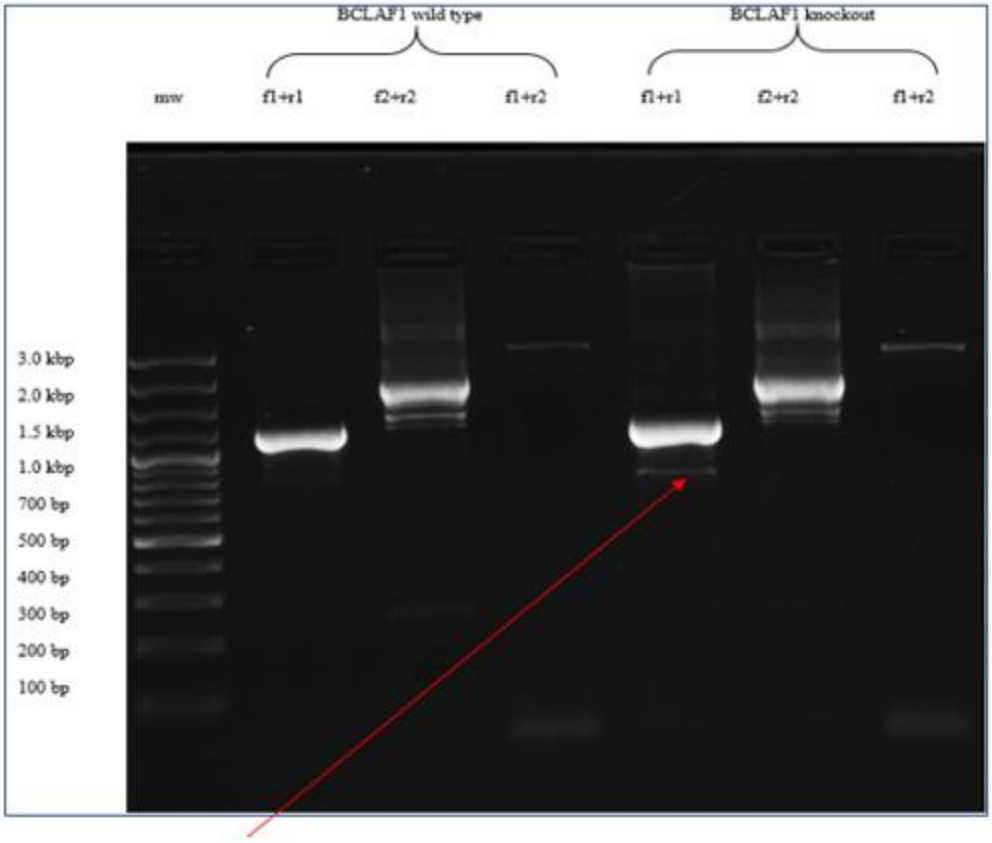

Figure 3.18 Agarose gel electrophoresis characterization of the *BCLAF1* amplified regions. Multiplex-PCR amplification products of the targeted regions of *BCLAF1* wild type and *BCLAF1* knockout cDNA using the primers described in the Materials and Methods table 4.1 were analysed on 1% agarose gel. mw: molecular weight marker.

Lane f1+r1 (primer set 1) product size is 1176 bp, lane f2+r2 (primer set 2) product size 1877bp and lane f1+r2 (primer set 1-forward primer + primer set 2-reverse primer) product size is 2999 bp.

Electrophoresis analysis of the multiplex-PCR products (**Figure 3.18**) showed the presence of the shorter, fainter band (900bp) heterozygous deletion in the exon 4 was observed in the *BCLAF1* KO sample; (f1 + r1 lane), pointed with the red arrow, but not in *BCLAF1* wild type sample. The CRISPR/Cas9-mediated *BCLAF1* knockout didn't work, hence, further analysis on the effects of *BCLAF1* knockout in cellular gene expression, signalling pathways and tumourigenesis was ceased.

3.3 Discussion

A tumour cell may contain many point mutations and various structural variants. The point mutations and structural variants such as insertions and deletions, translocations and copy number changes, loss of heterozygosity are few of the genetic alterations that are acquired during cancer progression (Albertson *et al.*, 2003; Popova *et al.*, 2009). Several genetic alterations disrupt the normal balance during cell proliferation, survival and differentiation and result in loss of control of cellular transformation (Hahn *et al.*, 1999; Mitelman *et al.*, 2007). Understanding the genetics mechanisms of cancer contribute to identification of new cancer genes, tumour classification and cancer genome analysis (Garraway & Lander, 2013; Liu *et al.*, 2015).

BCLAF1 aberrant events have been observed in various tumours and its deficiency has been associated with tumour development (Kasof *et al.*, 1999; Mwapagha, 2014). *BCLAF1* plays a key role in several cellular pathways, including apoptosis, RNA splicing, transcriptional regulation, DNA damage response, lung development and T-cell activation (Kasof *et al.*, 1999; McPherson *et al.*, 2009; Sarras *et al.*, 2010; Lee, Yu, *et al.*, 2012; Sarras, 2012; Savage *et al.*, 2014). *BCLAF1* was originally identified as a proapoptotic and repressor of transcription through an interaction of anti-apoptotic bcl2 family members (Kasof *et al.*, 1999). Several subsequent studies have elaborated more extensively on the role of *BCLAF1* in regulation of apoptotic genes and induction of apoptosis (Sarras *et al.*, 2010; Lee, Yu, *et al.*, 2012). Since *BCLAF1* is involved γH2AX-mediated DNA repair and RNA splicing and transcript stabilization of genes involved in DDR pathways it is suggested that *BCLAF1* is a tumour suppressor (Lee, Yu, *et al.*, 2012; Savage *et al.*, 2014).

Here we used siRNA mediated gene knockdown studies to identify the contribution of *BCLAF1* to tumourigenesis, by examining the effects of *BCLAF1* deficiency in signalling pathways such as apoptosis, DDR and cell cycle. This study identified *H2AX* as a downstream gene of *BCLAF1* and its deficiency by siRNA downregulates H2AX protein expression.

We examined the downstream effects of *BCLAF1* deficiency on apoptotic genes. Our study indicated that *BCLAF1* deficiency downregulates the expression of proapoptotic *BAX* and *Caspase-3* but not *Caspase-9* in human fibroblast CT1 cells. These findings are consistent with a role for *BCLAF1* in cell death signalling, highlighted in the studies by (Kasof *et al.*, 1999;

McPherson *et al.*, 2009; Lee, Yu, *et al.*, 2012) that *BCLAF1* is a death promoting protein and overexpression of *BCLAF1* induces apoptosis.

P53 is a major regulator of cell growth, apoptosis and dictates cell fate, depending on the severity of DNA damage. In the G1 phase cell cycle arrest, *P53* triggers transcription of $P21^{(cip1/waf1)}$ a key component in cell cycle regulation (Kruse & Gu, 2009). *BCLAF1* has long been shown to be involved in the transcription of *P53* by interacting with CPE-TP53 during DNA damage (Liu *et al.*, 2007), furthermore, (Lee, Yu, *et al.*, 2012) suggested a role of *BCLAF1* in *P21* regulation, and its deficiency upregulates *P21* expression. Our studies confirmed that knocking down *BCLAF1* significantly upregulates *P21* expression and attenuates *P53* expression, which leads to a *P21*-dependent G1 phase cell cycle arrest. Moreover, *BCLAF1* knockdown reduced apoptotic cells in the cell cycle flow, in agreement with our results on *BCLAF1* deficiency and downregulation of Caspase-3 and BAX protein levels.

In addition to the role of *BCLAF1* in DDR, (Savage *et al.*, 2014) also suggested *BCLAF1* as a key component of a DNA damage induced-BRCA1 protein complex that regulates pre-mRNA splicing of various of genes including EXO1, ATRIP and BACH1. These genes are involved in DNA damage signalling and repair, promoting the genomic stability (Caestecker & Van de Walle, 2013). Consistent with the role of *BCLAF1* in mRNA splicing, we observed significant effects of *BCLAF1* deficiency on *EXO1*, *ATRIP*, and *BACH1* mRNA transcripts in human fibroblast CT1 cells, and our results confirmed that *BCLAF1* is indeed required for *EXO1*, *ATRIP* and *BACH1* mRNA stabilization and efficient production of transcripts, and that abrogation of *BCLAF1* reduced the expression of these genes. We have also examined DNA repair dynamics in *BCLAF1*-depleted CT1 cells following DNA damage, previously highlighted by (Lee, Yu, *et al.*, 2012; Savage *et al.*, 2014) studies, our study supports these findings and shows that *BCLAF1* depleted cells showed a significant defect in their ability to resolve γH2AX-marked DNA lesions 5 hours post UV treatment, which is consistent with our finds of *BCLAF1* knockdown downregulation of H2AX protein levels, hence less activity of γH2AX induced DNA repair 5 hours post irradiation resulting in an increased DNA lesions compared to control cells.

Although (Savage *et al.*, 2014) findings suggested that *BCLAF1* depleted cells exhibited defect in their ability to rectify γH2AX-marked DNA breaks 24 hours post irradiation, they supported their findings to *BCLAF1* role in BRCA1/BCLAF1 mRNA splicing complex, involved in mRNA

splicing and stabilization of genes required for DNA repair and maintenance of genomic stability. In addition, our studies suggested that the downregulation of H2AX in *BCLAF1* depleted cells contributes to their inability to resolve γH2AX-marked DNA lesions, hence elevated defective DNA repair in *BCLAF1* depleted cells in comparison to control cells.

Taken together, the contribution of *BCLAF1* to tumourigenesis involves different cancer signalling pathways, such as cell death signalling, cell growth and division and DDR signalling pathways and cancer associated *BCLAF1* variants deregulate *BCLAF1* downstream gene expression involved in the above mentioned cancer signalling pathways leading to uncontrolled cell growth, survival and accumulation of apoptosis-resistant cells, defective DNA repair and genomic instability, all events are hallmarks of cancer.

Chapter 4

Conclusions

The investigation of cancer molecular events is of immense importance, as it provides a distinct direction by which to fight cancer. This study set out to identify the role and contribution of *BCLAF1* in tumourigenesis. Transformed human lung fibroblasts, the CT1 cell line, was used a model to examine and understand the molecular role of *BCLAF1* knockdown in tumourigenesis.

BCLAF1 has previously been reported to be mutated in various tumours and differently expressed in various tumour types (Kasof *et al.*, 1999; Zhou *et al.*, 2014). In this study, we investigated the expression of *BCLAF1* in different human cancer cell lines. *BCLAF1* expression was found to be supressed in all human cancer cell lines examined. Extremely low levels of expression of *BCLAF1* was detected in the MDA-231 cell line, both at protein and RNA levels. Although *BCLAF1* deregulation alone may not be sufficient to initiate tumourigenesis, *BCLAF1* deregulation alters the expression of a number of downstream genes involved in apoptosis, damage DNA repair and cell cycle regulation. Hence, differential expression of genes downstream of *BCLAF1* was observed.

The functional consequences of *BCLAF1* on apoptosis, cell cycle, and damaged DNA repair and its contribution to carcinogenesis was demonstrated by the knockdown of *BCLAF1* gene expression in the CT1 cell line using RNA interference technology, as RNAi ensures the specific silencing of gene expression with no off-target effects. This study showed that *BCLAF1* knockdown attenuated the expression of apoptotic genes such as *BAX* and *Caspase-3* both at RNA and protein levels. However, antiapoptotic *BCL2* and *BCL-XL* expression in CT1 cells were largely unaffected when *BCLAF1* expression was inhibited. This study also determined that *H2AX* is a downstream gene of *BCLAF1* and that siRNA-mediated *BCLAF1* knockdown downregulates damaged DNA repair genes *EXO1*, *ATRIP*, *BACH1* as well as *H2AX* expression. In addition, our study suggested that the downregulation of *H2AX* in *BCLAF1* depleted cells contributes to their inability to resolve γH2AX-marked DNA sites, resulting in elevated defective DNA repair in *BCLAF1* depleted CT1 cells in comparison to control cells. The inhibition of *BCLAF1* resulted in the downregulation of *P53* and a slight accumulation of *P21*, resulting in a *P21*-depedent G1 cell cycle arrest (**Figure 4.1**).

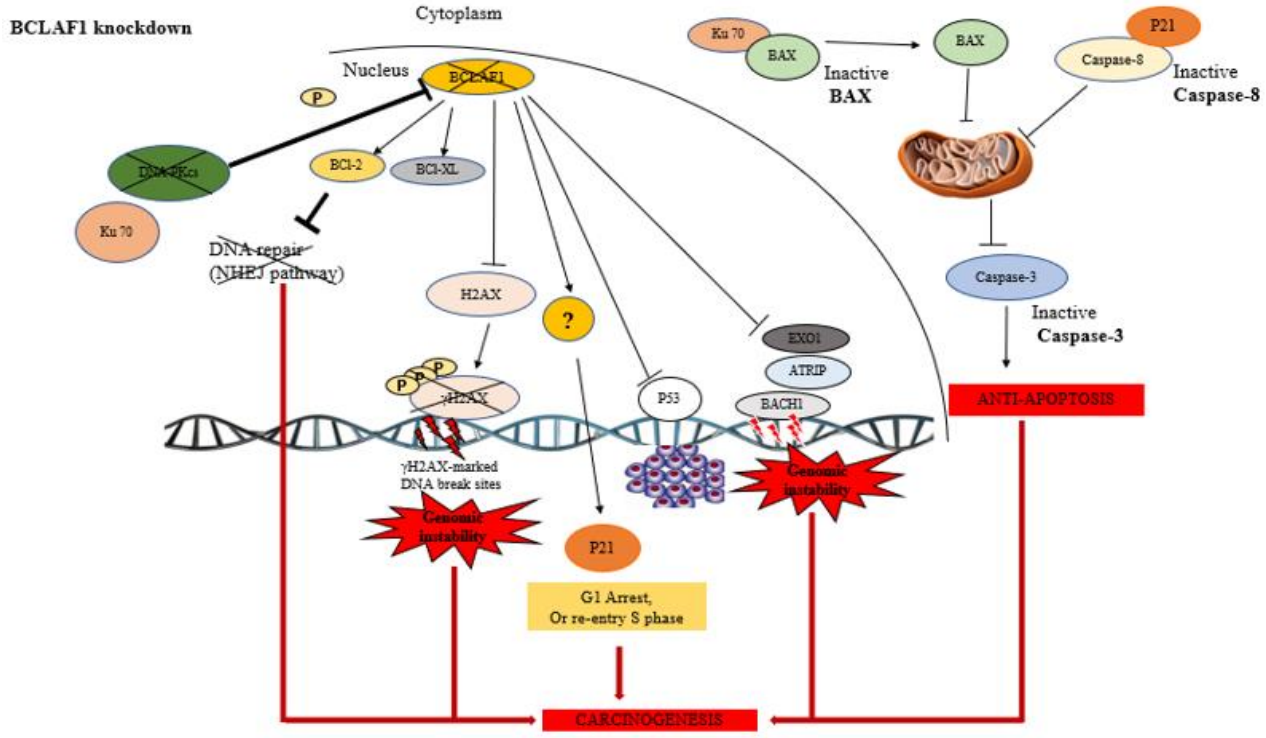

Figure 4.1 The downstream effects of *BCLAF1* knockdown. In the cytoplasm of siRNA-mediated *BCLAF1* knockdown cells, the expression of apoptotic genes such as *BAX* and *Caspase-3* is inhibited, which results in anti-apoptosis signals in cells. In the nucleus, the suppression of *BCLAF1* leads to weakened Ku70/DNA-PKcs complex but kept the expression of *BCL-2* and *BCL-XL* instead, subsequently reducing the activity of DSB repair. The depletion of *BCL-2* enhances the DNA binding activity of the Ku70/DNA-PKcs complex for accelerating DNA repair. The knockdown of *BCLAF1* downregulates the expression of *H2AX*, hence, reduced levels of γH2AX production contributing to cells inability to resolve γH2AX-marked DNA break sites. This results in elevated defective DNA repair and genomic instability. Consistent with the role of *BCLAF1* in mRNA splicing of DNA repair genes EXO1, ATRIP, BACH1, depletion of *BCLAF1* attenuated/inhibits the expression of *EXO1*, *ATRIP*, *BACH1*. *BCLAF1* knockdown results in the downregulation of *P53*, subsequently resulting in *P53*-dependent uncontrolled growth of cells. Furthermore, *BCLAF1* deficiency upregulates *P21*, through an unknown P21-promoter, resulting in a *P21*-depedent G1 cell cycle arrest. Modified from (Lee, Yu, *et al.*, 2012).

Taken together, these results show that abrogation of *BCLAF1* expression in CT1 cells can result in dysregulation of several cancer signalling pathways. Moreover, *BCLAF1* deficiency resulted in *P53*-dependent uncontrolled growth of cells, survival and accumulation of apoptosis-resistant cells, defective DNA repair and genomic instability; all events that are crucial in tumourigenesis.

Chapter 5

Materials and methods

5.1 Cell lines and cell culture

5.1.1 Cell lines

Transformed immortalized keratinocyte cell line, HaCaT; lung transformed fibroblasts, CT1; cervical cancer cell line, HeLa; breast cancer cell line, MDA-231 and two oesophageal cancer cell lines; KYSE30 and WHCO1 were used in the study and CT1 was used as model cell line for transfections in this study. HeLa (ATCC® CCL-2™), MDA-231 (ATCC® HTB-26™) and HaCaT (the ATCC number is no longer available) cell lines are from the American Type Culture Collection (ATCC); CT-1 was a gift from (Namba *et al.*, 1980), WHCO1 and KYSE30 was a gift from (Veale, 1984).

5.1.2 Cell culture and maintenance

Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% (P/S) penicillin/streptomycin (complete medium) in a humidified environment and 5% CO2 at 37˚C. The medium was replaced every 2 to 3 days by aspirating the used medium and washing the cells with 10ml of Phosphate Buffered Saline (PBS) and fed with fresh complete medium. Cells were sub-cultured every 4 to 5 days, by removing the medium, rinsing with PBS incubating in 0.05% trypsin-10 mM EDTA in PBS for 3 minutes. Once the cells had detached, the trypsin- was inactivated by adding 5 ml of complete medium. Cells were pelleted by centrifugation at 4000 rpm for 5 minutes, re-suspended in 3 ml of complete medium and 1 ml aliquots were re-plated in fresh plates. For the preparation of frozen stocks, the cells were re-suspended in cell freezing medium (70 % DMEM, 20 % FBS and 10 % DMSO) by gently pipetting. The cells were stored in liquid nitrogen.

5.2 Genetic interference and modification

5.2.1 RNA interference

For the inhibition of gene expression, siRNA was used, *BCLAF1* siRNA (EHU069911-20UG) was obtained from Sigma Aldrich. siRNA consisting of a scrambled sequence (SIC001, Sigma Aldrich) was used as a non-silencing control. Cells in DMEM containing 10% FBS without antibiotics were transiently transfected with 20nM siRNA using MISSION® siRNA Transfection Reagent (S1452-100µL, Sigma Aldrich). RNA and Protein were harvested 24 hours post-transfection (as described

in Sections 5.3.1 and 5.4.1 respectively) and the downstream effects of cellular gene expression were examined by qRT-PCR and Western Blot analysis respectively (as described in Sections 5.3.2 and 5.4.2). The effect of the respective siRNAs on cell cycle and DNA repair was determined using the flow cytometry and immunofluorescence assays respectively. Briefly, cells were seeded either on cover slips or directly into 6-well tissue culture plates for immunofluorescence and flow cytometry respectively, transfected with 20 nM siRNA and incubated for 24 hours. The effect of *BCLAF1* siRNA on cell cycle and DNA repair was assayed using the flow cytometry and immunofluorescence reagents respectively.

5.2.2 CRISPR/Cas9 *BCLAF1* gene editing transfection

CT1 cells were cultured in DMEM containing 10% FBS without antibiotics in 24-well plates at a density of 100,000 cells per well, 24 hrs before transfection. The cells were transiently transfected with the recommended combination of mKate2-Cas9 expression plasmid DNA (100 µg/µL), (Dharmacon Inc., #U-004100-120), CRISPR-Cas9 synthetic tracrRNA (10 µM) , (Dharmacon Inc., #U-002000-20), and two human *BCLAF1* crRNAs (5 nM each), (Dharmacon Inc.), targeting exon 4; target sequence: GAGGAATGAGACGACCTTAT, genomic location and PAM: hg38|-chr6:136278665 GGG and exon 7; target sequence: CGAAGTGAACCGCTCGTTTA, genomic location and PAM: hg38|+chr6:136273138-136273160 GGG using DharmaFECT Duo transfection reagent (Dharmacon Inc., #T-2010-03) as per the manufacturer's protocols. Cells were incubated for 72 hours before proceeding with cell sorting and gene editing studies.

5.2.3 Fluorescence-activated cell sorting (FACS)

72 hours post transfection, the old medium was aspirated and trypsinized the cells in 0.5 ml of 0.05% trypsin-EDTA as described above in Section 5.1.2. The cell pellet was collected by centrifugation at 4000 rpm for 3 minutes, re-suspended in 2 ml of DMEM in 5 ml falcon round-bottom tubes for FACS analysis.

Table 5.1 PCR primer sequences

Primer	Sequence		Product size
Primer set 1	FW	5'- AGA TCG GAA GTG ACG GAG -3'	1176 bp
	RV	5'- CAT TCT GTG GTG CGA TTG TC -3'	
Primer set 2	FW	5'- TTC ATC ACA TCC CTT CAC G -3'	1877 bp
	RV	5'- TTC CTT GCG TCT GTC CTT C -3'	
Primer set 1-Forward primer + Primer set 2-Reverse primer	FW	5'- AGA TCG GAA GTG ACG GAG -3'	2999 bp
	RV	5'- TTC CTT GCG TCT GTC CTT C -3'	

5.3 RNA Extraction and quantification and PCR analysis

5.3.1 RNA Extraction and quantification

RNA was extracted from cells using QIAzol Lysis Reagent (QIAGEN, Hilden, Germany). The medium was aspirated, and cells were washed with cold PBS. Appropriate volume of QIAzol respective to the size of the plate was added onto the cells (1 ml for 10 cm plates and 0.5 ml for 6-well plates). The cells were lysed by scrapping them off the plate using the cell scrapper and transferred to 1.5 ml Eppendorf tubes. The homogenized sample was incubated for 5 minutes at room temperature. 200 µl of chloroform was added shaken vigorously for 15 seconds to mix well and incubated for 3 minutes at room temperature. The samples were then centrifuged at 14000 rpm for 15 minutes at 4°C. The upper aqueous layer was transferred to a fresh labelled tube and 0.5 ml of isopropanol was added, mixed thoroughly by shaking (do not vortex) for 15 seconds and incubated at room temperature for 10 minutes. The samples were then centrifuged at 14000rpm for 20 minutes at 4°C to pellet the RNA. The supernatant was carefully removed and added 1ml 75% DEPC-ethanol and vortexed on low for 5-10 seconds to wash the pellet thoroughly, centrifuged at 12000 rpm for 5 minutes at 4°C to re-pellet the RNA. The supernatant was carefully removed, and air dried at room temperature for 10 minutes. The pellet was then dissolved in 20-30 µl DEPC treated-water by gentle pipetting and incubated at 55°C for 5 minutes. RNA was quantitated on a NanoDrop 2000/2000c Spectrophotometer (Thermo Scientific, IL, USA) and stored at -80°C.

5.3.2 cDNA synthesis and qRT-PCR analysis

For first strand cDNA synthesis, total RNA was reverse-transcribed using ImProm-II Reverse Transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions as indicated in (Table 5.2). 5 µg template mRNA, 1 µl oligo dT and dH2O were mixed to a final volume of 9 µl. This mixture was heated for 10 minutes at 70 ˚C to denature any secondary structure in the RNA followed by annealing to the oligo dT primers. The mixture was chilled on ice for 5 minutes. The second master mix (Table 5.3) of MgCl2, 5x first strand synthesis buffer, dNTPs mix, RNAse inhibitor and reverse transcriptase were added and incubated for 2 hours at 42°C, followed by 10 minutes at 70°C to inactivate the reverse transcriptase.

Table 5.2 cDNA synthesis master mix 1

5µg mRNA	Oligo dT	ddH_20	Total
Y	1	X	9 µl

Table 5.3 cDNA synthesis master mix 2.

Reagents	**Volume (µl)**
5X first strand synthesis buffer	5
$dNTP_s$ mix	1
RNase inhibitor	1
$MgCL_2$	2
Impromp II Reverse Transcriptase	1
ddH2O	1
Total	9

One microliter of the cDNA was subsequently amplified using the KAPA SYBR qPCR Master Mix (KAPA SYBR Fast qPCR Kit, KAPA Biosystems) on a Roche Lightcycler 480 II. Briefly, to a mixture of SYBR Green PCR Master Mix, 20 µM of forward and reverse primers, as well as 1µl of cDNA was added, in a total volume of 10 µl (Table 5.4). GAPDH was used as a control.

Table 5.4. qRT-PCR mixture set up for each gene

Reagents	**Volumes (µl)**
SYBR Green PCR Master Mix	5
Forward primer (20 uM)	1
Reverse primer (20 uM)	1
ddH_2O	2
cDNA	1
Total	10

Table 5.5 Sequences of primers used for real-time RT-PCR analysis

Primer	Sequence	Annealing Temperature (°C)
BCLAF1	FW 5'- GGG GCG TAT TGA TGT TGA AAG -3' RV 5'- GAG CAC GAG GAC AGA CTA AAC -3'	60
BAX	FW 5'- GGT TGT CGC CCT TTT CTA CT -3' RV 5'- AAG TCC AAT GTC CAG CCC AT -3'	
Caspase-3	FW 5'- ACA TGA CTC AGC CTG TTC C -3' RV 5'- GCC TCA CCA CCT TTA GAA C -3'	
Caspase-9	FW 5'- GTG AAC TTC TGC CGT GAG TC -3' RV 5'- GCA AAG CCA GCA CCA TTT TC-3'	
BCL2	FW 5'- CTG CAC CTG ACG CCC TTC ACC -3' RV 5'- CAC ATG ACC CCA CCG AAC TCA AAG -3'	
BCL-XL	FW 5'- GAT CCC CAT GGC AGC AGT AAA GCA AG-3' RV 5'- CCC CAT CCC GGA AGA GTT CAT TCA CT -3'	
P53	FW 5'- CTG CTC AGA TAG CGA TGG TCT G -3' RV 5'- TTG TAG TGG ATG GTG GTA CAG TCA -3'	
P21	FW 5'- ACC TCA CCT GCT CTG CTG C -3' RV 5'- ATT AGG GCT TCC TCT TGG AGA -3'	
EXO1	FW 5'- CTC AAG TGG GAG AGG CTT TG -3' RV 5'- AAC GCT GTC CTG GAA GAG AA -3'	
ATRIP	FW 5'- AGG CTG CTA ACC TCT GTC GGA -3' RV 5'- CAG AGA CTC CCA GCA AGG TC -3'	
BACH1	FW 5'- CTG CCA CCT CCC AAC ATA GT-3' RV 5'- GAG ATG CAG CAC AGA CCA AA -3'	
Ku70	FW 5'- GGC TGT GGT GTT CTA TGG -3' RV 5'- CCC TTA AAC TGG TCA AGC -3'	
H2AX	FW 5'-TCC CTT CCA GCA AAC TCA AC-3' RV 5'- CCA TCT AAA ACT CCC CAA TGC-3'	
BRCA1	FW 5'-CAT GCT GAA ACT TCT CAA CCA-3' RV 5'-TGT AGG CTC CTT TTG GTT ATA T-3'	
GAPDH	FW 5'- GGCTCTCCAGAACATCATCC - 3' RV 5'- GCCTGCTTCACCACCTTC- 3'	

5.3.3 Preparation of a 1% agarose gel and electrophoresis

1% Agarose gels were prepared by boiling 5g agarose powder in 500 ml 1X TBE electrophoresis buffer. After the solution had cooled down to~42°C, 30 µl of ethidium bromide (0.5 µg/ml) was added to the solution and mixed properly. The gel was poured into the gel tank, combs were inserted and allowed to set and solidify at room temperature. 1X TBE electrophoresis buffer was added to the tank and used as the running buffer. The total of 10 µl cDNA was mixed with 3 µl of DNA loading buffer and the mixture was run on 1% agarose gel. Electrophoresis was done at 100 V for 90minutes. The gels were then digitally photographed using UVP BioSpectrum Imaging System (UVP, USA).

5.4 Protein extraction and Western blot analysis

5.4.1 Protein extraction

Cells were grown to 80% confluency, washed with cold PBS and lysed in RIPA buffer and the culture plates were incubated on ice on a shaker for 30 minutes. A cell scraper was used to dislodge the cells. The lysate was transferred to a 1.5 ml tube and sonicated once for 10 seconds (Heat System-Ultrasonics, USA). The lysate was centrifuged at 4°C for 15 minutes at 14000 rpm and the supernatant was collected, leaving the pellet consisting of insoluble cell debris. Protein concentrations were quantified using the BCA protein assay kit (Thermo Scientific, USA), according to manufacturer's instruction. Briefly, a standard curve with concentrations between 0 and 2000 µg/ml was constructed using Bovine Serum Albumin (BSA). 200 µl of working reagent was added to the standards and samples in a 96-well plate. The plate was incubated for 30 minutes at 37°C, and the absorbance was measured at 595 nm using the Multiskan FC plate reader (Thermo Scientific, USA). To determine the protein concentrations, the equation of the slope of the standard curve was used.

5.4.2 Western blot analysis

Proteins were separated on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE). The SDS PAGE gels were made with 4% stacking gel and 10% separating gel (**Table 5.7** and **Table 5.6**) respectively. Equal protein concentrations (30 µg) were heat denatured at 95°C for 5 minutes in 1x loading dye, cooled and loaded into their respective wells. A PAGE Ruler Plus Prestained Protein Ladder (Thermo Scientific, USA) was loaded to determine the size of the protein bands (10-250 kDa) of interest and electrophoresed at 100 V for 2 hours. Proteins were

transferred to ECL™ nitrocellulose membrane in 1X transfer buffer for 90 minutes at 4°C. The membranes were rinsed thrice for 10 minutes in 10 ml TBST on a shaker at room temperature. To reduce nonspecific binding, membranes were blocked in 10 ml 5% non-fat milk in TBST for 1 hour on a shaker at room temperature, following the incubation of membranes at 4°C overnight on a shaker with primary antibodies. Western blot analyses were performed using the rabbit polyclonal anti-BTF (ab107177), rabbit polyclonal anti-caspase-3 (phospho S150) (ab59425), rabbit polyclonal anti-Caspase-9 (ab209495), rabbit monoclonal anti-BAX (ab182733), rabbit polyclonal anti-Bcl-2 (ab115807), rabbit polyclonal anti-Exonuclease 1 (ab106303), rabbit polyclonal anti-gamma H2AX (phospho S139) (ab11174), rabbit polyclonal anti-p53 (ab17990), rabbit polyclonal anti-p21 (ab219811), and rabbit polyclonal anti-BTF (ab107177) primary antibodies (Abcam Biotechnology company). The following day, membranes were washed thrice for 10 minutes in 10 ml TBST at room temperature, then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 hour on a shaker. The protein of interest was detected using the Lumiglo Reserve™ Chemiluminescent Substrate Kit (KPL, USA). The UVP BioSpectrum Imaging System (UVP, USA) was used to visualise the protein bands of interest.

The primary and secondary antibodies were stripped off the membranes by washing once with 10 ml 1M Glycine pH 2.5, then a wash with 10 ml Tris pH 7.5 and thrice using 10 ml TBST. The membranes were then incubated with 10 ml 5% non-fat milk in TBST for 1 hour before re-probing with different primary antibodies.

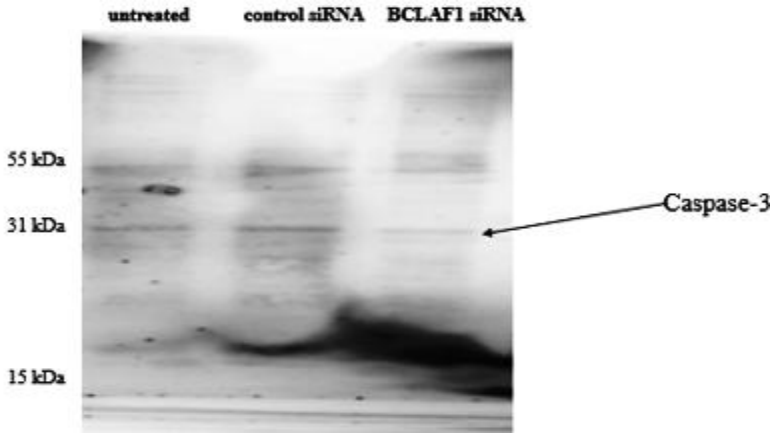

Figure 5.1 Caspase-3 blot gel. Both untreated, control siRNA and *BCLAF1* siRNA data for Caspase-3 are from the same gel.

Table 5.6 Proteins molecular weight

Protein	Molecular weight (kDa)
BCLAF1	106
GAPDH	37
BAX	21
Caspase-3	31
Caspase-9	46
BCL-2	26
H2AX	15
EXO1	115
P53	53
P21	32

5.5 Cell cycle analysis

For cell cycle analyses, CT1 cells were plated in 6-well plates, transfected with 20 nM siRNA and incubated for 24 hours (as described in Section 5.2.1). Cells were harvested and 800 µl of ice cold absolute ethanol was added to fix the cells before storage at -20°C for a day. The samples were thawed at room temperature, centrifuged at 4000 rpm for 5 minutes and the ethanol was decanted carefully. The pellet was resuspended in 1 ml PBS and the tube was centrifuged at 4000 rpm for 5 minutes. The PBS was discarded and 100 µl of RNAse solution (1:20 dilution of a 10 µg/ml solution in PBS) was pipetted into each tube followed by incubation at 37°C for 15 minutes. 500 µl of propidium iodide (1:100 dilution in PBS) was added to each sample for 30 minutes before proceeding to visualise and analyse the samples using the FACScan Cell Sorter (Benton Dickinson, NJ, USA) and the Cellquest and Modfit (Benton Dickinson, NJ, USA) analysis software.

5.6 Apoptosis assays

To assess the effects of *BCLAF1* siRNA effects on apoptosis, apoptosis detection assay was performed using FITC Annexin V Apoptosis Detection Kit, (BD Pharmingen™) according to the manufacturer's instruction. The following controls were used to set up compensation and quadrants: unstained cells, cells stained with Annexin V-FITC (no PI), cells stained with PI (no

Annexin V-FITC). The cells were properly gated dual parameter dot plots of X-axis, FITC fluorescence versus Y-axis, PI fluorescence has been shown in logarithmic fluorescence intensity. Differentiation of cell population was done on flow cytometer using the FACScan Cell Sorter (Benton Dickinson, NJ, USA). The apoptotic activity of each sample was measured 24 hr after *BCLAF1* siRNA transfection.

5.7 UV exposure and DNA damage validation

The medium was carefully removed from each well by tilting the 6-well plate and aspirating from the edge. Cells were exposed to different doses ($0J/M^2$, $40J/M^2$, $200J/M^2$, $500J/M^2$) of UV light. DNA was extracted from each sample by adding 1ml of SDS -Tris-EDTA (SDS-TE) (1 ml of 10% SDS in 10 mM TE buffer pH 8) buffer to cells, following the addition of 10 µl of proteinase K (100 µg/ml). The mixture was incubated at 50°C overnight. The following day, RNA was removed, and the DNA denatured by the addition of 66 µl of 5M NaOH and incubation at 37°C for 1 hour. 40 µl of the DNA extracts were ran on a 1% agarose gel at 100 V for 90 minutes for DNA damage validation.

5.8 Immunofluorescence analysis for *BCLAF1* siRNA effects on DNA repair

24 hours post *BCLAF1* siRNA transfection, the medium was carefully removed from cells plated on cover slips and the medium placed in 12ml falcon tubes separately. Cells were exposed to $40J/M^2$ and placed back the old media containing the transfection complexes onto the cells. At the indicated time points, cells were permeabilized in absolute methanol for 5 minutes at -20°C and immediately fixed at room temperature for 5 minutes with 4% paraformaldehyde. After three rinses with PBS, blocking solution (1% BSA, 0.5% TritonX-100 in PBS) was added for 1 hour. 100 µl of *BCLAF1* (*BCLAF1* knockdown validation) or γH2AX (DNA pair activity) primary antibodies (1:500 dilution) were added and the coverslips were incubated overnight at 4°C in a humidified chamber. After three washes with PBS for 10 minutes, goat anti rabbit IgG-CFL 488 (sc-362262) secondary antibodies (1:1000 dilution) were added and incubated for 90 minutes at room temperature in a dark humidified chamber. Antibodies used were rabbit polyclonal anti-BTF (ab107177), rabbit polyclonal anti-gamma H2AX (phospho S139) (ab11174). The coverslips were washed three times with PBS, counter stained with 100µl 0.5ug/ml DAPI (DNA stain) for 10 minutes then rewashed once with 10ml PBS at room temperature. Flouromount aqueous Mounting Medium (Sigma-Aldrich Cat. No. F4680) was used to mount the coverslip upside down on the

glass slide and the slides were stored at room temperature in the dark overnight and placed at 4°C until viewing under the Olympus 1X81 Inverted Fluorescent microscope using the cellSens Dimension Software (Olympus Imaging Software).

5.9 Statistical analysis

All experiments were performed in triplicate. For all data comparisons, the Student's t test was performed using GraphPad prism. A P value of <0.05 was considered statistically significant and *p < 0.05, **p < 0.01, ***p < 0.001.

5.10 Solutions and buffers

5.10.1 Protein solutions

RIPA buffer

25 ml 1 M Tris pH 7.4

5 ml NP-40

2.5 g Na-deoxycholate

0.5 g SDS

15 ml NaCl

2 ml 0.5M EDTA

1.05 g NaF

Dissolve in 300 ml dH2O and dilute to 500 ml with dH2O

10% Separating Gel

Table 5.7 10% Separating gel recipe

	X1
ddH2O	2.72 ml
1M Tris Buffer pH 8.8	3.75 ml
30% Acrylamide	3.3 ml
10% SDS	100 µl
10% APS	100 µl
Tetramethylethylenediamine (TEMED)	50 µl

4% Stacking Gel

Table 5.8 4% Stacking gel recipe

	X1
ddH2O	3.65 ml
1M Tris Buffer pH 6.8	0.625 ml
30% Acrylamide	0.650 ml
10% SDS	50 µl
10% APS	25 µl
Tetramethylethylenediamine (TEMED)	5 µl

1 M Glycine pH 2.5

Add 75 g Glycine in 800 ml of ddH2O, pH 2.5 using HCl.

Adjust the volume to 1 L with ddH2O

Autoclave.

10X Running Buffer

30 g Tris base

144 g Glycine

10.0 g SDS

Dilute to 1 litre with dH2O

1X Running Buffer

100 ml 10X Running buffer

Bring to 1 L with ddH2O

10X Transfer Buffer

30 g Tris base

144.1 g Glycine

Dilute to 1 litre with dH2O

1X Transfer Buffer

100 ml 10x Transfer buffer

200 ml methanol

Bring to 1 L with ddH2O

4 M NaCl

Dissolve 292.2 g NaCl in a final volume of 1 L ddH2O

TBST

250 µl Tween 20

30 ml 5 M NaCl

50 ml 1 M Tris pH 7.5

Dilute to 1 litre with dH2O

4% non-fat milk blocking solution

2.5 g non-fat milk powder

Dissolve in 50 ml with TBST

4X protein loading dye

2.5 ml 1M Tris pH 6.8

4 ml 20% SDS

0.2 ml 0.1% Bromophenol Blue

4 ml Glycerol

50 µl β-mercaptoethanol

Blocking solution for immunofluorescence microscopy

0.5 g BSA

50 µl Triton X-100

Dilute to 50 ml with PBS

10% Ammonium Persulfate (APS)

Ammonium persulfate 5 g

ddH2O 50 ml

Store at -20°C

1 M Tris pH 7.5

Add 121 g Tris base to 800 ml ddH2O

Adjust pH with concentrated HCl to pH 7.5

Add distilled water up to one litre

Autoclave.

1 M Tris pH 6.8

Add 121 g Tris base to 800 ml ddH2O

Adjust pH with concentrated HCl to pH 6.8

Add distilled water up to one litre

Autoclave.

1 M Tris pH 8.8

Add 121 g Tris base to 800 ml ddH2O

Adjust pH with concentrated HCl to pH 8.8

Add distilled water up to one litre

Autoclave.

Glycine pH 2.5

10% sodium dodecyl sulphate (SDS)

Dissolve 10 g sodium dodecyl sulphate in 80 ml distilled water (heat to 80°C),

Bring to 100 ml with distilled water

5.10.2 RNA and DNA solutions

DEPC water

1 ml DEPC in 1 litre dH2O.

Incubate for 1 hour at 30°C and autoclave to remove the DEPC.

75 % DEPC- Ethanol

75 ml absolute ethanol

25 ml DEPC-treated water.

0.5 µg/ml DAPI stain

5 ul 0.5 mg/ml DAPI stock solution in 4.5 ml PBS

0.5 M EDTA, pH 8.0

Add 18.61 g of Na2EDTA.2H2O to 80 ml ddH2O

Adjust the pH to 8.0 with 10 NaOH

Add distilled water to a final volume of 100 ml

Autoclave and store at room temperature.

5 M NaOH

Dissolve 20.0 g of NaOH pellets in 80 ml ddH2O

When cooled, bring to the final volume 100 ml.

10X TBE buffer

Dissolve 108 g Tris in 800 ml

Add 55 g Boric Acid (Mix)

Add 40 ml 0.5 Na2EDTA

Adjust volume to 1 L, store at room temperature.

1% Agarose gel

5 g of Agarose powder

500 ml of 1X TBE buffer

5.10.3 Tissue culture solutions

70 % Ethanol

70 ml of absolute ethanol

Add 30 ml of distilled water

Freezing media

70 % Fetal bovine serum

20 % DMEM media

10 % Dimethylsulphoxide (DMSO)

PBS (pH 7.4)

154 mM NaCl

2.7 mM KCl

5.6 mM Na_2HPO4 or $Na_2HPO_42H_2O$

1 mM KH_2PO_4

Adjust pH to 7.4 using concentrated HCl or NaOH. Bring to final volume and autoclave.

References

Abbas, T., & Dutta, A. (2009). p21 in cancer: intricate networks and multiple activities. *Nature Reviews Cancer, 9*(6), 400–414.

Aizawa, R., Matsumoto, S., Uneno, Y., Nishikawa, Y., Ozaki, Y., Mori, Y., Kanai, M., Ishida, Y., Sakanaka, K., & Hiraoka, M. (2017). Severe esophagitis associated with cytomegalovirus during concurrent chemoradiotherapy for esophageal cancer. *Japanese Journal of Clinical Oncology, 47*(9), 885-888. doi:10.1093/jjco/hyx083

Albertson, D. G., Collins, C., McCormick, F., & Gray, J. W. (2003). Chromosome aberrations in solid tumors. *Nature Genetics, 34*(4), 369-376. doi:10.1038/ng1215

Barrangou, R., Birmingham, A., Wiemann, S., Beijersbergen, R. L., Hornung, V., & Smith, A. v. B. (2015). Advances in CRISPR-Cas9 genome engineering: lessons learned from RNA interference. *Nucleic Acids Research, 43*(7), 3407-3419.

Boyer, A. P., Collier, T. S., Vidavsky, I., & Bose, R. (2013). Quantitative proteomics with siRNA screening identifies novel mechanisms of trastuzumab resistance in HER2 amplified breast cancers. *Molecular & Cellular Proteomics, 12*(1), 180-193.

Bracken, C. P., Wall, S. J., Barré, B., Panov, K. I., Ajuh, P. M., & Perkins, N. D. (2008). Regulation of cyclin D1 RNA stability by SNIP1. *Cancer Research, 68*(18), 7621-7628.

Brown, G. T., Cash, B., Alnabulsi, A., Samuel, L. M., & Murray, G. I. (2016). The expression and prognostic significance of bcl-2-associated transcription factor 1 in rectal cancer following neoadjuvant therapy. *Histopathology, 68*(4), 556-566.

Ca´ ceres, J. F., Misteli, T., Screaton, G.R., Spector, D.L., and Krainer, A.R. (1997). Role of the modular domains of SR proteins in subnuclear localization and alternative splicing specificity. *Journal of Cell Biology, 138*(2), 225-238.

Caestecker, K. W., & Van de Walle, G. R. (2013). The role of BRCA1 in DNA double-strand repair: past and present. *Experimental Cell Research, 319*(5), 575-587.

Chang, F., Syrjänen, S., Wang, L., & Syrjänen, K. (1992). Infectious agents in the etiology of esophageal cancer. *Gastroenterology, 103*(4), 1336-1348.

Chin, K., DeVries, S., Fridlyand, J., Spellman, P. T., Roydasgupta, R., Kuo, W. L., Lapuk, A., Neve, R. M., Qian, Z., Ryder, T., Chen, F., Feiler, H., Tokuyasu, T., Kingsley, C., Dairkee, S., Meng, Z., Chew, K., Pinkel, D., Jain, A., Ljung, B. M., Esserman, L., Albertson, D. G., Waldman, F. M., & Gray, J. W. (2006). Genomic and transcriptional aberrations linked to breast cancer pathophysiologies. *Cancer Cell, 10*(6), 529-541. doi:10.1016/j.ccr.2006.10.009

Degterev, A., Boyce, M., & Yuan, J. (2003). A decade of caspases. *Oncogene, 22*(53), 8543-8567.

Downs, J. A., & Jackson, S. P. (2004). A means to a DNA end: the many roles of Ku. *Nature Reviews Molecular Cell Biology, 5*(5), 367-378.

El-Shinawi, M., Mohamed, H. T., El-Ghonaimy, E. A., Tantawy, M., Younis, A., Schneider, R. J., & Mohamed, M. M. (2013). Human cytomegalovirus infection enhances NF-κB/p65 signaling in inflammatory breast cancer patients. *PLoS One, 8*(2), e55755. doi: 10.1371/journal.pone.0055755

Elbendary, A. A., Cirisano, F. D., Evans, A., Davis, P. L., Iglehart, J., Marks, J. R., & Berchuck, A. (1996). Relationship between p21 expression and mutation of the p53 tumor suppressor gene in normal and malignant ovarian epithelial cells. *Clinical Cancer Research, 2*(9), 1571-1575.

Fuchs, Y., & Steller, H. (2011). Programmed cell death in animal development and disease. *Cell, 147*(4), 742-758.

Garcia, A. I., Buisson, M., Bertrand, P., Rimokh, R., Rouleau, E., Lopez, B. S., Lidereau, R., Mikaélian, I., & Mazoyer, S. (2011). Down-regulation of BRCA1 expression by miR-146a and miR-146b-5p in triple negative sporadic breast cancers. *EMBO Molecular Medicine, 3*(5), 279-290.

Garraway, L. A., & Lander, E. S. (2013). Lessons from the cancer genome. *Cell, 153*(1), 17-37.

Giancotti, F. G. (2014). Deregulation of cell signaling in cancer. *FEBS Letters, 588*(16), 2558-2570.

Giménez-Bonafé, P., Tortosa, A., & Pérez-Tomás, R. (2009). Overcoming drug resistance by enhancing apoptosis of tumor cells. *Current Cancer Drug Targets, 9*(3), 320-340.

Goossens, N., Nakagawa, S., Sun, X., & Hoshida, Y. (2015). Cancer biomarker discovery and validation. *Translational Cancer Research, 4*(3), 256.

Griffiths, A., Miller, J., & Suzuki, D. (2000). *An Introduction to Genetic Analysis. WH Freeman New York* (7th ed.).

Guicciardi, M. E., & Gores, G. J. (2009). Life and death by death receptors. *The FASEB Journal, 23*(6), 1625-1637.

Guo, W., Chen, W., Yu, W., Huang, W., & Deng, W. (2013). Small interfering RNA-based molecular therapy of cancers. *Chinese Journal of Cancer, 32*(9), 488-493.

Hahn, W. C., Counter, C. M., Lundberg, A. S., Beijersbergen, R. L., Brooks, M. W., & Weinberg, R. A. (1999). Creation of human tumour cells with defined genetic elements. *Nature, 400*(6743), 464-468.

Hahn, W. C., & Weinberg, R. A. (2002). Rules for making human tumor cells. *New England Journal of Medicine, 347*(20), 1593-1603.

Hainaut, P., & Plymoth, A. (2013). Targeting the hallmarks of cancer: towards a rational approach to next-generation cancer therapy. *Current Opinion in Oncology, 25*(1), 50-51.

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell, 100*(1), 57-70.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell, 144*(5), 646-674.

Haussecker, D. (2016). Stacking up CRISPR against RNAi for therapeutic gene inhibition. *The FEBS Journal, 283*(17), 3249-3260.

Heaney, A. P., & Melmed, S. (2005). *Oncogenes and Tumor Suppressor Genes in Tumorigenesis of the Endocrine System Endocrinology* (pp. 301-309). doi:10.1007/978-1-59259-829-8

Herbein, G., & Kumar, A. (2014). The oncogenic potential of human cytomegalovirus and breast cancer. *Frontiers in Oncology, 4*(230). doi:10.3389/fonc.2014.00230

Herrero, A. B., Rojas, E. A., Misiewicz-Krzeminska, I., Krzeminski, P., & Gutiérrez, N. C. (2016). Molecular mechanisms of p53 deregulation in cancer: an overview in multiple myeloma. *International Journal of Molecular Sciences, 17*(12), 2003.

Jin, J., Hu, C., Wang, P., Chen, J., Wu, T., Chen, W., Ye, L., Zhu, G., Zhang, L., & Xue, X. (2014). Latent infection of human cytomegalovirus is associated with the development of gastric cancer. *Oncology Letters, 8*(2), 898-904.

Jurel, S. K., Gupta, D. S., Singh, R. D., Singh, M., & Srivastava, S. (2014). Genes and oral cancer. *Indian Journal of Human Genetics, 20*(1), 4-9.

Kasof, G. M., Goyal, L., & White, E. (1999). Btf, a novel death-promoting transcriptional repressor that interacts with Bcl-2-related proteins. *Molecular and cellular biology, 19*(6), 4390-4404.

Kaufmann, K. B., Büning, H., Galy, A., Schambach, A., & Grez, M. (2013). Gene therapy on the move. *EMBO Molecular Medicine, 5*(11), 1642-1661.

Khan, F. A., Pandupuspitasari, N. S., Chun-Jie, H., Ao, Z., Jamal, M., Zohaib, A., Khan, F. A., Hakim, M. R., & ShuJun, Z. (2016). CRISPR/Cas9 therapeutics: a cure for cancer and other genetic diseases. *Oncotarget, 7*(32), 52541–52552.

Kong, S., McBurney, M. W., & Fang, D. (2012). Sirtuin 1 in immune regulation and autoimmunity. *Immunology and Cell Biology, 90*(1), 6-13.

Kruse, J.-P., & Gu, W. (2009). Modes of p53 regulation. *Cell, 137*(4), 609-622.

Lawrence, M. S., Stojanov, P., Mermel, C. H., Robinson, J. T., Garraway, L. A., Golub, T. R., Meyerson, M., Gabriel, S. B., Lander, E. S., & Getz, G. (2014). Discovery and saturation analysis of cancer genes across 21 tumour types. *Nature, 505*(7484), 495-501. doi:10.1038/nature12912

Lee, S. H., Kalejta, R. F., Kerry, J., Semmes, O. J., O'Connor, C. M., Khan, Z., Garcia, B. A., Shenk, T., & Murphy, E. (2012). BclAF1 restriction factor is neutralized by proteasomal degradation and microRNA repression during human cytomegalovirus infection. *Proceedings of the National Academy of Sciences, 109*(24), 9575-9580.

Lee, Y. Y., Yu, Y. B., Gunawardena, H. P., Xie, L., & Chen, X. (2012). BCLAF1 is a radiation-induced H2AX-interacting partner involved in gammaH2AX-mediated regulation of apoptosis and DNA repair. *Cell Death and Disease, 3*, e359. doi:10.1038/cddis.2012.76

Liu, B., Conroy, J. M., Morrison, C. D., Odunsi, A. O., Qin, M., Wei, L., Trump, D. L., Johnson, C. S., Liu, S., & Wang, J. (2015). Structural variation discovery in the cancer genome using next generation sequencing: computational solutions and perspectives. *Oncotarget, 6*(8), 5477-5489.

Liu, H., Lu, Z. G., Miki, Y., & Yoshida, K. (2007). Protein kinase C delta induces transcription of the TP53 tumor suppressor gene by controlling death-promoting factor Btf in the apoptotic response to DNA damage. *Molecular and Cellular Biology, 27*(24), 8480-8491. doi:10.1128/MCB.01126-07

Lowe, S. W., & Ruley, H. (1993). Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. *Genes & Development, 7*(4), 535-545.

Luo, Q., Beaver, J. M., Liu, Y., & Zhang, Z. (2017). Dynamics of p53: A Master Decider of Cell Fate. *Genes, 8*(2), 66.

Marshall, C. J. (1991). Tumor suppressor genes. *Cell, 64*(2), 313-326.

Marx, D., Binder, C., Meden, H., Lenthe, T., Ziemek, T., Hiddemann, T., Kuhn, W., & Schauer, A. (1997). Differential expression of apoptosis associated genes bax and bcl-2 in ovarian cancer. *Anticancer Research, 17*(3C), 2233-2240.

Marx, D., & Meden, H. (2001). *Differential expression of apoptosis-associated genes Bax and Bcl-2 in ovarian cancer* Vol. 39. *Ovarian Cancer: Methods and Protocols* (pp. 687-691). doi:10.1385/1-59259-071-3:687.

McPherson, J. P. (2012). BCLAF1 (BCL2-associated transcription factor 1). *Atlas of Genetics and Cytogenetics in Oncology and Haematology, 15*(12), 994-997. doi:10.4267/2042/46079

McPherson, J. P., Sarras, H., Lemmers, B., Tamblyn, L., Migon, E., Matysiak-Zablocki, E., Hakem, A., Azami, S. A., Cardoso, R., & Fish, J. (2009). Essential role for Bclaf1 in lung development and immune system function. *Cell Death & Differentiation, 16*(2), 331-339.

Merup, M., Moreno, T. C., Heyman, M., Rönnberg, K., Grandér, D., Detlofsson, R., Rasool, O., Liu, Y., Söderhäll, S., & Juliusson, G. (1998). 6q Deletions in Acute Lymphoblastic Leukemia and Non-Hodgkin9s Lymphomas. *Blood, 91*(9), 3397-3400.

Merz, C., Urlaub, H., Will, C., & Lührmann, R. (2007). Protein composition of human mRNPs spliced in vitro and differential requirements for mRNP protein recruitment. *RNA, 13*, 116–128.

Michaelis, M., Doerr, H. W., & Cinatl Jr, J. (2009). The story of human cytomegalovirus and cancer: increasing evidence and open questions. *Neoplasia, 11*(1), 1-9.

Mitelman, F., Johansson, B., & Mertens, F. (2007). The impact of translocations and gene fusions on cancer causation. *Nature Reviews Cancer, 7*(4), 233-245.

Morgan-Lappe, S., Woods, K., Li, Q., Anderson, M., Schurdak, M., Luo, Y., Giranda, V., Fesik, S., & Leverson, J. (2006). RNAi-based screening of the human kinome identifies Akt-cooperating kinases: a new approach to designing efficacious multitargeted kinase inhibitors. *Oncogene, 25*(9), 1340-1348.

Murray, A. W. (2004). Recycling the Cell Cycle:Cyclins Revisited. *Cell Press, 116*, 221–234.

Mwapagha, L. M. (2014). *The role of viral sequences in genetic aberrations and malignant transformation.* (Doctor of Philosophy), University of Cape Town.

Namba, M., Nishitani, K., & Kimoto, T. (1980). Characteristics of WI-38 cells (WI-38 CT-1) transformed by treatment with Co-60 gamma rays. *GANN Japanese Journal of Cancer Research, 71*(3), 300-307.

Nickles, D., Falschlehner, C., Metzig, M., & Boutros, M. (2012). A genome-wide RNA interference screen identifies caspase 4 as a factor required for tumor necrosis factor alpha signaling. *Molecular and Cellular Biology, 32*(17), 3372-3381.

Orr, K. S., & Savage, K. I. (2015). The BRCA1 and BRCA2 Breast and Ovarian Cancer Susceptibility Genes—Implications for DNA Damage Response, DNA Repair and Cancer Therapy *Advances in DNA Repair*: InTech.

Pistritto, G., Trisciuoglio, D., Ceci, C., Garufi, A., & D'Orazi, G. (2016). Apoptosis as anticancer mechanism: function and dysfunction of its modulators and targeted therapeutic strategies. *Aging (Albany NY), 8*(4), 603-619. doi:10.18632/aging.100934.

Popova, T., Manié, E., Stoppa-Lyonnet, D., Rigaill, G., Barillot, E., & Stern, M. H. (2009). Genome Alteration Print (GAP): a tool to visualize and mine complex cancer genomic profiles obtained by SNP arrays. *Genome Biology, 10*(11), R128.

Radia, A.-M., Yaser, A.-M., Ma, X., Zhang, J., Yang, C., Dong, Q., Rong, P., Ye, B., Liu, S., & Wang, W. (2013). Specific siRNA targeting receptor for advanced glycation end products (RAGE) decreases proliferation in human breast cancer cell lines. *International Journal of Molecular Sciences, 14*(4), 7959-7978.

Saitoh, N., Spahr, C. S., Patterson, S. D., Bubulya, P., Neuwald, A. F., & Spector, D. L. (2004). Proteomic analysis of interchromatin granule clusters. *Molecular Biology of the Cell, 15*(8), 3876-3890.

Sánchez-Rivera, F. J., & Jacks, T. (2015). Applications of the CRISPR-Cas9 system in cancer biology. *Nature Reviews Cancer, 15*(7), 387-395.

Sarras, H. (2012). *A Role for Bclaf1 in mRNA Processing and Skeletal Muscle Differentiation.* (Doctor of Philosophy), University of Toronto.

Sarras, H., Alizadeh Azami, S., & McPherson, J. P. (2010). In search of a function for BCLAF1. *The Scientific World Journal, 10*, 1450-1461.

Savage, K. I., Gorski, J. J., Barros, E. M., Irwin, G. W., Manti, L., Powell, A. J., Pellagatti, A., Lukashchuk, N., McCance, D. J., & McCluggage, W. G. (2014). Identification of a BRCA1-mRNA splicing complex required for efficient DNA repair and maintenance of genomic stability. *Molecular Cell, 54*(3), 445-459.

Shao, A., Sun, H., Geng, Y., Peng, Q., Wang, P., Chen, J., Xiong, T., Cao, R., & Tang, J. (2016). Bclaf1 is an important NF-κB signaling transducer and C/EBPβ regulator in DNA damage-induced senescence. *Cell Death & Differentiation, 23*(5), 865-875.

Soroceanu, L., & Cobbs, C. S. (2011). Is HCMV a tumor promoter? *Virus Research, 157*(2), 193-203.

Soussi, T., Dehouche, K., & Béroud, C. (2000). p53 website and analysis of p53 gene mutations in human cancer: forging a link between epidemiology and carcinogenesis. *Human Mutation, 15*(1), 105-113.

Talseth-Palmer, B. A., & Scott, R. J. (2011). Genetic variation and its role in malignancy. *International Journal of Biomedical Science: IJBS, 7*(3), 158-171.

Tsujimoto, Y., Gorham, J., Cossman, J., Jaffe, E., & Croce, C. M. (1985). The t (14; 18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. *Science, 229*, 1390-1394.

Umemoto, K., Kojima, Y., Nagata, N., Yokoi, C., Sakurai, T., Kobayakawa, M., Iizuka, T., Igari, T., Yanase, M., & Akiyama, J. (2016). Cytomegalovirus esophagitis developing during chemoradiotherapy for esophageal cancer: two case reports. *Journal of Medical Case Reports, 10*(1), 259. doi:10.1186/s13256-016-0947-y

Utrera-Barillas, D., Valdez-Salazar, H.-A., Gómez-Rangel, D., Alvarado-Cabrero, I., Aguilera, P., Gómez-Delgado, A., & Ruiz-Tachiquin, M.-E. (2013). Is human cytomegalovirus associated with breast cancer progression? *Infectious Agents and Cancer, 8*(1), 12. doi:10.1186/1750-9378-8-12.

Valcárcel, J., & Green, M. R. (1996). The SR protein family: pleiotropic functions in pre-mRNA splicing. *Trends in Biochemical Sciences, 21*(8), 296-301.

Veale, A. (1984). Atypical cytokeratins synthesized by human oesophageal carcinoma cells in culture. *South African Journal of Science, 80*(6), 260.

Vogelstein, B., & Kinzler, K. W. (1992). p53 function and dysfunction. *Cell, 70*(4), 523-526.

Vohhodina, J., Barros, E. M., Savage, A. L., Liberante, F. G., Manti, L., Bankhead, P., Cosgrove, N., Madden, A. F., Harkin, D. P., & Savage, K. I. (2017). The RNA processing factors THRAP3 and BCLAF1 promote the DNA damage response through selective mRNA splicing and nuclear export. *Nucleic Acids Research, 45*(22), 12816-12833.

Wang, Q., Gao, F., May, W. S., Zhang, Y., Flagg, T., & Deng, X. (2008). Bcl2 negatively regulates DNA double-strand-break repair through a nonhomologous end-joining pathway. *Molecular Cell, 29*(4), 488-498.

Welcsh, P. L., & King, M.-C. (2001). BRCA1 and BRCA2 and the genetics of breast and ovarian cancer. *Human Molecular Genetics, 10*(7), 705-713.

White, E. (1996). Life, death, and the pursuit of apoptosis. *Genes & Development, 10*(1), 1-15.

White, E. (2001). Regulation of the cell cycle and apoptosis by the oncogenes of adenovirus. *Oncogene, 20*(54), 7836-7846. doi:10.1038/sj.onc.1204861

White, E., & Cipriani, R. (1990). Role of adenovirus E1B proteins in transformation: altered organization of intermediate filaments in transformed cells that express the 19-kilodalton protein. *Molecular and Cellular Biology, 10*(1), 120-130.

Yadav, S. S., Li, J., Lavery, H. J., Yadav, K. K., & Tewari, A. K. (2015). *Next-generation sequencing technology in prostate cancer diagnosis, prognosis, and personalized treatment.* Paper presented at the Urologic Oncology: Seminars and Original Investigations.

Yip, K., & Reed, J. (2008). Bcl-2 family proteins and cancer. *Oncogene, 27*(50), 6398-6406.

Zhou, X., Li, X., Cheng, Y., Wu, W., Xie, Z., Xi, Q., Han, J., Wu, G., Fang, J., & Feng, Y. (2014). BCLAF1 and its splicing regulator SRSF10 regulate the tumorigenic potential of colon cancer cells. *Nature Communications, 5*(5), 4581. doi:10.1038/ncomms5581.

Ziegelbauer, J. M., Sullivan, C. S., & Ganem, D. (2009). Tandem array-based expression screens identify host mRNA targets of virus-encoded microRNAs. *Nature Genetics, 41*(1), 130-134. doi:10.1038/ng.266